## REMARKS

Amendments to the claims have been made to respond to the issues and concerns raised in the Office Action, to clarify aspects in the specification and claims, and to refine claim language. The amendments are believed to be consistent with the disclosure originally filed. The amendments also have been particularly presented to avoid, where applicable, any admission or estoppel, generally, negatively affecting the scope of protection provided by the disclosure and claims of the present application, and also in a manner that avoids prosecution history estoppel, limitation of the scope of equivalences, or the like. Any amendment should not be construed as an admission regarding the propriety of any objection or rejection raised in any Office Action, and the Applicant reserves the right to pursue the full scope of the unamended claims in any subsequent patent application as may be appropriate.

Claim 28 has been amended. Claims 1-27, 29, 41-44, and 47-48 have been cancelled. Claims 28, 30-40, 45-46, and 49-50 remain in the application. Each amendment is believed to have been made in accordance with Rule 121. However, should any unintended informality exist, it is requested that the undersigned be contacted by telephone so that it may be resolved as expediently as possible. It is believed the amendments fully respond to the issues raised in the Office Action. Further detail with respect to specific points raised in the Office Action is offered below.

Included with this response is an Information Disclosure Statement. While the Information Disclosure Statement includes additional information for the Office to consider and may increase the examination burden associated with this Application, the Applicant believes the Information Disclosure Statement is the only way for it to comply with its obligations under Rule 56.

The Office has expressed concerns regarding Applicant's priority claims. The Applicant disagrees that the instant application is not entitled to the priority dates as

claimed. Applicant's claims are supported by at least provisional application 60/211,093, filed June 12, 2000.

Aspects relating to managing a plurality of female bovine mammals for a reproductive factor may be found, *inter alia*, at page 1, first full paragraph ("By early weaning calves from spring calving cows, the dams have the ability to increase BCS prior to winter and decrease cost of feed without detrimental effects on calf performance"); page 2, first full paragraph ("Sexed semen is a relatively new technology that grants the IS ability to perpetuate the program using the female progeny of the initial heifers", "…AI using low dose sexed semen resulted in pregnancy rates in heifers that were 70-90% of unsexed traditional dose controls"); page 2, ending paragraph continued to page 3 ("Behavior and physiological indicators monitored puberty…"); page 3, first full paragraph ("Estrus synchronization of the EW heifers was accomplished…", "Heifers were AI'd following standing heat up to 72 hr…", "… fixed-time mating of all remaining pubertal heifers occurred", "A 45 d breeding period allowed heifers three opportunities to be AI"); page 3 second full paragraph ("The IS produces all female progeny to perpetuate the system in subsequent years by utilizing sexed semen…"); page 4, first full paragraph ("…gains are attributed to adjusted feed rations to allow for gains that would induce early puberty", "Once the heifers began to cycle, the ration was backed down to avoid over-fattening of heifers and possible negative impacts on subsequent reproduction/calving difficulty"); page 4, second full paragraph ("This induction of early puberty is contributed to nutrition allowing greater gain and weight of the EW heifers", "At time of PGF injection…"); page 4, third full paragraph ("Heifers that became impregnated to sexed semen…"); page 4, fourth full paragraph ("…accomplished satisfactory gain performance of heifers which enabled induction of early puberty and resulted in 9 mo old heifers impregnated to sexed semen", "the opportunity for three services of AI may result in adequate overall pregnancy rate"), page 7 ("…the IS may include early weaning, estrous synchronization, and AI", "Sexed semen can be utilized to yield female progeny", "…induce early puberty by 9 mo of age", "…greater gains and weights of heifers induced early puberty…", "…resulted in 9 mo old heifers pregnant to sexed semen").

Aspects relating to inducing early puberty in said plurality of female bovine mammals may be found, *inter alia*, at page 2, carryover paragraph from page 1 ("… faster gains resulting in younger puberty age", "Early puberty permits early insemination…"); page 4, first full paragraph ("…gains are attributed to adjusted feed rations to allow for gains that would induce early puberty"); page 4, second full paragraph ("This induction of early puberty is contributed to nutrition allowing greater gain and weight of the EW heifers", "At time of PGF injection, the number of EW heifers cycling increased to 85% with only 7 heifers pre-pubertal"); page 4, fourth full paragraph ("…accomplished satisfactory gain performance of heifers which enabled induction of early puberty…"); page 7 ("…to induce early puberty by 9 mo of age", "… greater gains and weights of heifers induced early puberty").

Aspects relating to inseminating substantially all of the female bovine mammals with sex-sorted spermatozoa may be found, *inter alia*, at page 2, first full paragraph ("…AI using low dose sexed semen resulting in pregnancy rates…"); page 3, second full paragraph ("The IS produces all female progeny to perpetuate the system in subsequent years by utilizing sexed semen"); page 4, third full paragraph ("Heifers that became impregnated to sexed semen…"); page 4, fourth full paragraph ("…and resulted in 9 mo old heifers impregnated to sexed semen"), page 7 ("…resulted in 9 mo old heifers pregnant to sexed semen").

Aspects relating to producing offspring comprising substantially all female offspring may be found, *inter alia*, at page 2, first full paragraph ("Sexed semen is a relatively new technology that grants the IS ability to perpetuate the program using the female progeny of the initial heifers"); page 3, second full paragraph ("The IS produces all female progeny…"); page 7 ("Sexed semen can be utilized to yield female progeny, decrease calving difficulty risk and create a second generation to perpetuate the IS").

Aspects relating to harvesting substantially all of said plurality of female bovine mammals may be found, *inter alia*, at page 1, second full paragraph ("…a program

designed to allow a heifer to produce one calf prior to harvest", "…in the SCH these animals are profitable because of faster growth during feedlot period and larger, more profitable carcasses", "…heifers to reach harvest weight by 30 months"); page 1, ending paragraph continued on page 2 ("…produce a calf by 18 mo of age and a carcass by 24 mo of age", "… the period from weaning to harvest is shortened").

In sum, each of the steps cited in the Office Action is disclosed in the Applicant's priority cases and support the method of managing female bovine mammals as claimed in the current case. The Applicant respectfully requests the Office recognize Applicant's priority claims.

The Office has raised definiteness concerns with respect to managing a plurality of bovine female mammals for at least one reproductive factor. The Applicant disagrees that managing an animal for a reproductive factor is unclear. The concepts and terminology related to reproductive management of animals is well known to practitioners in the field, as evidenced by example in the discussion of this concept and terminology in the Pursel (*see* pages 301-305), Bagnato (*see* pages 874-875, 878), Pankowski (*see* pages 1477-1479), Scipioni (*see* pages 1742, 1744), and Fricke (*see* pages 1918, 1922-1925) publications included with this response as Exhibit "A". Moreover, the Applicant disagrees that the relationship of this step to the remainder of the recited steps is unclear. The remainder of the recited steps each are concerned with effecting reproduction of the bovine female animals that are the subject of the claims, and therefore are the natural product of the reproductive management step discussed. Accordingly, the Applicant respectfully requests the Office withdraw its definiteness concerns.

The Office has raised definiteness concerns with respect to unsexed spermatozoa. The Applicant disagrees that this term has no art recognized definition. This term is well known to practitioners in the field, as evidenced by example in multiple usages of the term in each of the Ferre (*see* Abstract 266), Grant (*see* page 185), Seidel (*see* pages 738-740), Garner (*see* pages 522, 524), Tubman (*see* pages 1029-1030), and Weigel (*see*

pages E120-E129) publications included with this response as Exhibit "B". The discussion in Weigel may be particularly extensive. Moreover, the Applicant disagrees the specification does not define what is intended to be encompassed by a typical number of unsexed spermatozoa. First, Applicant notes the complete recitation of claim 45 is "a number of spermatozoa from about 10% to about 50% relative to a typical number of unsexed spermatozoa". This complete recitation is defined in the specification at page 10, lines 16-22. Additionally, Applicant notes the specification at page 10, lines 4-5 incorporates by reference U.S. Patent Application 09/001,394, now U.S. Patent No. 6,149,867, and at page 4, lines 13-14 incorporates by reference published international patent application WO 99/33956. Each of these documents sets forth a clear definition of a typical number, in the case of the U.S. patent at column 12, lines 27-49, and in the case of the published international patent application, at page 19, lines 6-19. Accordingly, the Applicant respectfully requests the Office withdraw its definiteness concerns.

The Office raises obviousness concerns with respect to various combinations of references that rely on Ereth (Proceedings of the Western Section, 2000) and Ereth (Journal of Animal Science, 2000). The Applicant submits that the Ereth references may not be used by the Office to support an obviousness rejection. Ereth (Proceedings of the Western Section, 2000) was published in the Proceedings, Western Section, American Society of Animal Science, Vol. 51, 2000. The publication date of this document is June 21, 2000, as evidenced by the cover pages and table of contents page attached to this response as Exhibit "C". Ereth (Journal of Animal Science, 2000) was published in the Journal of Animal Science, Vol. 78, Supplement 2, 2000. The publication date of this document is June 21, 2000, and indeed was received by at least one university library as late as June 27, 2001, each as evidenced by the pages from the journal attached to this response as Exhibit "D". By comparison, Applicant's priority date for the current claims is June 12, 2000. Accordingly, the present application predates the Ereth references cited by the Office. In addition, the Applicant notes that author B.A. Ereth is the same person as Applicant Barbi A. Riggs. Given this fact and the other common authorship of the Ereth references and the present application, the Applicant anticipates being able to disqualify the Ereth references through use of a *Katz*-type declaration pursuant to MPEP

§ 715.01(c), if necessary. Accordingly, the Applicant respectfully requests the Office withdraw its obviousness concerns with respect to all combinations relying on the Ereth references.

The Office raises obviousness concerns with respect to various combinations of references that rely on Hohenboken. Hohenboken did not actually accomplish any of Applicant's claimed steps. Rather, Hohenboken merely speculates that such steps may be possible. *See e.g.* Hohenboken at page 1428 ("*Theoretical* calculations by Taylor et al. (20) *suggested*…", "…biological efficiency of beef production *would* be increased…", "Sex-ratio control *would* allow…", "*If* sexed semen were used…" [emphasis added]). Because Hohenboken merely discusses these concepts generally, and because Hohenboken does not present any empirical evidence of accomplishing a herd management system in practice, the teachings of Hohenboken at best merely make these concepts obvious to try. *See* MPEP 2145; *In re O'Farrell*, 853 F.2d 894 (Fed. Cir. 1988).

For example, the mere suggestion by Hohenboken that sperm sexing may find application in a herd management system falls short of actually achieving such sperm sexing in a herd management system in practice. Utilizing sperm sexing in a herd management system in practice may require accounting for variables not addressed by Hohenboken such as physical and chemical handling of sperm cells, speed and rate of sorting, and the like. At the time of Hohenboken, sperm sexing may have been a relatively new technology. What was obvious was merely to explore a new technology or general approach that seemed to be a promising field of experimentation. *See e.g.* MPEP 2145; *O'Farrell* at 903. Hohenboken gives only general guidance as to the particular form of Applicant's claimed invention or how to achieve it. *Id.*

Similarly, the mere suggestion by Hohenboken of allowing parturition at a younger age falls short of actually achieving such parturition at a younger age. The Office points to Petit at page 158 and Hall at page 1607 as teaching methods of inducing early puberty. However, Petit states the issue only in general terms, discussing an "improved level of nutrition" and "improved feeding". *See* Petit at page 1607. In Hall,

the highest documented weight gain was .82 kg/d, and the earliest documented onset of puberty was at 9.5 months for only 6 of 75 heifers. *See* Hall at page 1607 and Table 3. Neither Petit nor Hall actually bred nor harvested any of the animals. In contrast to Petit and Hall, the Applicant's procedure permitted weight gain of approximately 1.6 kg/d, induction of early puberty by 9 months, breeding by 10 months, and harvest prior to 24 months. *See* specification at page 13, lines 14-17; page 19, lines 6-8; page 51, lines 15-16. Even considering Petit and Hall, the nonspecific suggestion of Hohenboken of allowing parturition at a younger age amounts merely to a suggestion to vary all parameters or try each of numerous possible choices for inducing early puberty until one possibly arrives at a successful result. *See e.g.* MPEP 2145; *O'Farrell* at 903. Petit and Hall give no indication of which parameters are critical nor any direction as to which of many possible choices is likely to be successful in the manner of the Applicant. *Id.*

Because the disclosure of Hohenboken does not actually teach Applicant's invention and at best would merely make it obvious to try for Applicant's invention, the Applicant respectfully requests the Office to withdraw its obviousness concerns with respect to all combinations relying on Hohenboken.

The Applicant has amended claim 28 to change producing an offspring prior to the typical age of puberty to fertilizing prior to the typical age of puberty.

Having addressed each of the concerns raised in the Office Action, the Applicant respectfully requests reconsideration and withdrawal of the rejections and objections to the application. Allowance of claims 28, 30-40, 45-46, and 49-50 is requested at the Office's earliest convenience.

Dated this 12th day of July, 2007.

Respectfully submitted,
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| | |
|---|---|
| Application Number: | 09/879,480 |
| Filed: | June 12, 2001 |
| Title: | Integrated Herd Management System Utilizing Isolated Populations of X-Chromosome Bearing and Y-Chromosome Bearing Spermatozoa |

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# Exhibit A

# Reproductive Management of Lactating Dairy Cows Using Synchronization of Ovulation

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## ABSTRACT

Lactating dairy cows have poor reproductive efficiency because of low fertility and low rates of estrus detection. To eliminate the dependence on detection of estrus, we have recently developed a timed artificial insemination (AI) protocol that synchronizes the time of ovulation using GnRH and $PGF_{2\alpha}$. The effectiveness of this protocol as a management tool was compared with standard reproductive management. Lactating dairy cows (n = 333) from three herds were randomly assigned at parturition to two groups. Control cows were managed according to the typical reproductive strategy of the farm that relied on detection of estrus, the a.m.–p.m. breeding rule, and periodic use of $PGF_{2\alpha}$. Treated cows had timed AI after synchronization of ovulation with GnRH and $PGF_{2\alpha}$. For both groups, the voluntary waiting period was 50 d postpartum. Pregnancy diagnosis was performed by ultrasound between 32 and 38 d post-AI. Nonpregnant cows were inseminated again using the original treatment until diagnosed as pregnant or until culled from the herd. Median days to first AI (54 vs. 83) and days open (99 vs. 118) were lower for treated cows than for control cows, respectively. Pregnancy rates for the first AI were similar (37% vs. 39%) for the two groups even though treated cows were bred at an earlier time postpartum. More treated cows than control cows were pregnant at 60 d (37% vs. 5%) and at 100 d (53% vs. 35%) after calving. Thus, this protocol allowed effective management of AI in lactating dairy cows without the need for estrus detection. (**Key words**: reproduction, gonadotropin-releasing hormone, prostaglandin $F_{2\alpha}$, synchronized ovulation)

## INTRODUCTION

Increased milk production and increased herd size have dramatically influenced the manner in which dairy producers manage reproduction. Approximately 70 to 80% of the dairy farms in the US currently use AI, and most use daily estrus detection and the a.m.–p.m. breeding rule to manage AI. However, because the rate of estrus detection is <50% in most herds (20), variation in days to first and subsequent AI can be quite large. This inefficiency results in longer than optimal calving intervals and increased culling rates.

Over the past 25 yr, researchers (8, 11) have developed reproductive management protocols that synchronize the time of estrus using $PGF_{2\alpha}$. Synchronization with $PGF_{2\alpha}$ was successful when cows were bred to a detected estrus, because estrus detection rates increased and management of AI was more efficient than daily detection of estrus (24). Nevertheless, this management tool still did not control the time of AI, because estrus detection continued to be necessary. When researchers examined timed AI after $PGF_{2\alpha}$ in lactating dairy cows, pregnancy rates per AI was substantially lower than those for AI after a detected estrus (1, 13, 23). Low pregnancy rates from timed AI using $PGF_{2\alpha}$ might be partially explained by the variation in time of ovulation with respect to time of AI. Much of the variation in time to ovulation was probably due to the variation in stage of growth of the preovulatory follicle at the time of $PGF_{2\alpha}$ injection (14). For example, if $PGF_{2\alpha}$ was injected when a dominant follicle was fully developed and functional (i.e., d 7 or 8 of the cycle), the time to estrus and the variation in the time to estrus were significantly less than if some dominant follicles were in the early stages of development [i.e., around d 10 of the cycle (14, 25)].

Pursley et al. (17) devised a method of timed AI using GnRH and $PGF_{2\alpha}$ in which ovulation was synchronized within an 8-h period. An injection of GnRH was administered at a random stage of the estrous cycle, and a new follicular wave and dominant follicle emerged after this first injection of GnRH. An injection of $PGF_{2\alpha}$ was administered 7 d later to regress the corpus luteum. Ovulation was synchronized by a second injection of GnRH given 2 d after $PGF_{2\alpha}$. In 20 cows, ovulation was synchronized within an 8-h period, 24 to 32 h after the second injection of GnRH (17). This synchronized ovulation was possible because preovulatory follicles were at a similar stage of growth and were responsive to LH at the time of the

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second GnRH injection. This precise synchrony of ovulation resulted in similar pregnancy rates per AI after timed AI compared with cows bred to a detected estrus (19). This study examined whether synchronization of ovulation without estrus detection could be an effective method to manage reproduction in lactating dairy cows compared with daily detection of estrus and the a.m.–p.m. breeding rule.

## MATERIALS AND METHODS

This trial was conducted on three southern Wisconsin dairy farms beginning in June 1993 and ending in April 1994. Primiparous and multiparous lactating dairy cows were randomly assigned at parturition to two treatment groups (control vs. treated). Control cows (n = 166) received typical reproductive management: detection of estrus with the a.m.–p.m. breeding rule with occasional use of $PGF_{2\alpha}$ and GnRH when determined to be appropriate by the herd veterinarian and manager. Cows in the treated group (n = 167) had ovulation synchronized as described herein and had timed AI on 1 d of the week. Treated cows received an initial injection of 100 $\mu$g of GnRH (Cystorelin; Sanofi Animal Health, Inc., Overland Park, KS) between 40 and 48 d postpartum, 10 d prior to scheduled AI. Seven days later, cows received 35 mg of $PGF_{2\alpha}$ (The Upjohn Co., Kalamazoo, MI) to regress corpora lutea. This 7-d period was sufficient to regress the accessory corpus luteum resulting from the initial GnRH injection (17, 19). Forty-eight hours later, cows received a second injection of 100 $\mu$g of GnRH to initiate ovulation of the dominant follicle that had developed after the first injection of GnRH. The AI was performed 20 to 24 h after the second injection of GnRH.

The voluntary waiting period for both groups of cows was 50 d. The AI was performed by herd personnel who were normally in charge of AI, and service sires were chosen by the herd manager as part of their normal program of reproduction and genetics.

Pregnancy diagnoses were performed for cows in both groups between 32 and 38 d post-AI using an Aloka 500 ultrasound machine (Corometrics Medical Systems, Wallingford, CT) with a 7.5-MHz probe. Pregnancy was confirmed by ultrasound detection of a fetal heart beat. If the ultrasonographer was unsure of the pregnancy, the cow was reevaluated 1 wk later.

Cows in each group received the same treatment until diagnosed pregnant, culled from the herd, or until death. Control cows that were observed in estrus >15 d after AI were assumed nonpregnant and were reinseminated. Nevertheless, all control cows, including those that had been reinseminated, were evaluated for pregnancy at 32 to 38 d after the initial AI. If cows in the control group were diagnosed open and had not been inseminated since the AI in question, the herd manager was informed of the pregnancy diagnoses to allow a prompt management decision concerning reinsemination.

Cows in the treated group that were observed in estrus between the time of AI and pregnancy diagnosis were not reinseminated, and any reproductive management decisions were delayed until after pregnancy evaluation at 32 d post-AI. If cows were not pregnant, they were treated again with the synchronization protocol beginning at 32 d post-AI. If the ultrasound technician was unsure of the diagnosis, cows received the initial injection of GnRH and were rechecked 1 wk later, prior to the injection of $PGF_{2\alpha}$.

Pregnancy rates for each AI were defined as the number of cows pregnant at 32 to 38 d post-AI divided by the total number of cows that received that AI.

### Statistical Analyses

Covariables that were considered in the analysis of pregnancy rates per AI included season, farm, parity, treatment by parity interaction, and farm by parity interaction. Only the effect of season was significant; therefore, pregnancy rates per AI were stratified by season and analyzed using the Mantel-Haenszel chi-square test. Time to AI outcomes were censored when cows became pregnant, were culled from the herd, or when cows died. Time to pregnancy outcomes were censored at the time of pregnancy or at 32 d prior to either being culled from the herd or death, because this time represented the last possible time that they could have had a detectable pregnancy. Time data were analyzed by Kaplan-Meier survival curve estimation and the log rank statistics. The $P$ values for the median were obtained by bootstrap methods (5). Survival curves for time to second AI were obtained from the convolution of the time to first AI and the time between the first and second AI; survival curves for time to third AI were obtained from the convolution of time to second AI and time between second and third AI (9). This approach was necessary to avoid bias that resulted from changes in the number of cows that were eligible for each successive AI. For example, cows that became pregnant after the first AI were excluded from the group that were evaluated at the second AI.

## RESULTS

### Time to First, Second, and Third AI

Figure 1 illustrates the difference between the times to first AI for the treated and control groups

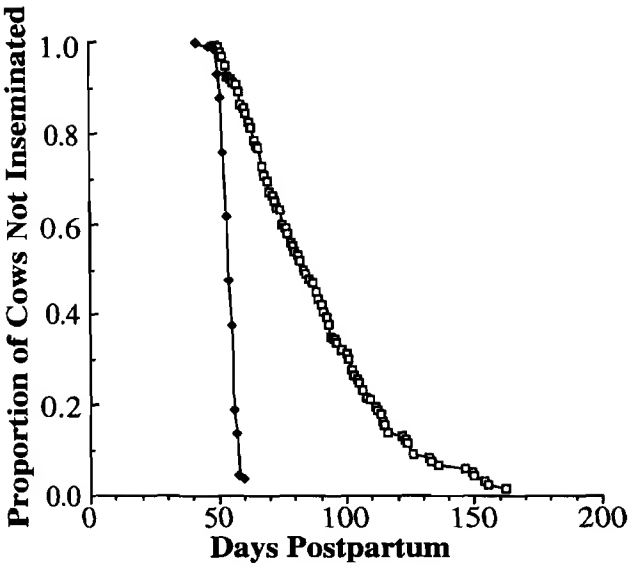


Figure 1. Survival curves for days to first AI in lactating Holstein cows managed with standard reproductive strategies (□) versus timed AI after synchronization of ovulation (♦).

using Kaplan-Meier curve for survival estimates. Because the synchronization protocol allowed the scheduling of AI with respect to the voluntary waiting period, the median days to first AI were less ($P < 0.001$) for treated cows than for control cows (Table 1). In addition, the range of days to first AI was less variable ($P < 0.001$) for cows in the treated group, 50 to 58 d, than for cows in the control group, 50 to 162 d (Figure 1). Also, times to second and to third AI were less ($P < 0.001$ and $P < 0.01$, respectively) for cows in the treated group than for cows in the control group (Table 1). The intervals among first, second, and third AI were similar for control and treated cows. The mean interval between breedings was about 42 d, although the nonpregnant cows in the treated group could not be reinseminated until 42 d after AI, and nonpregnant control cows were reinseminated at all detected estruses (Table 1).

### Pregnancy Rates per AI

Synchronization of ovulation and timed AI resulted in similar pregnancy rates at first AI compared with standard reproductive management (Table 1). In addition, pregnancy rates per AI were similar for second (Table 1) and third AI between treated and control cows.

### Days to Conception

Similar pregnancy rates per AI combined with less variation and fewer days to first and subsequent AI in the treated group led to a decrease ($P < 0.05$) in median days open (Figure 2). The survival curves indicated a greater likelihood for treated cows to be pregnant than for control cows to be pregnant at all times postpartum ($P < 0.001$ by log rank statistic). The median days to conception were less ($P < 0.05$) for treated cows than for control cows (Table 1). In addition, more treated than control cows were pregnant at 60 and 100 d postpartum ($P < 0.01$).

### Culling Rates

Of the cows in this study, more treated cows were culled than control cows that received AI (Table 2). Also, of the cows that received at least one AI, mean days postpartum on the day of culling were greater for control cows than for treated cows. No difference ($P > 0.05$) was detected in the number of cows culled from the control group (n = 35) compared with those culled from the treated group (n = 25).

## DISCUSSION

The present study demonstrated that the use of a protocol that allows effective timed AI can improve

TABLE 1. Median days postpartum (PP) at AI, pregnancy rate (PR) per AI, and median days to conception in lactating Holstein cows[1] inseminated after a detected estrus (control) or time inseminated after synchronization of ovulation (treated).

| | AI | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | First | | Second | | Third | | Pregnant | | Median interval |
| Treatment | PP | PR | PP | PR | PP | PR | 60 d PP | 100 d PP | to conception |
| | (d) | (%) | (d) | (%) | (d) | (%) | (%) | | (d) |
| Control | 83 | 39 | 128 | 45 | 170 | 61 | 5 | 35 | 118 |
| Treated | 54 | 37 | 96 | 42 | 140 | 48 | 37 | 53 | 99 |
| *P* | <0.001 | >0.25 | <0.001 | >0.15 | <0.001 | >0.15 | <0.01 | <0.01 | <0.05 |

[1]Cows per AI: first AI (n = 298), second AI (n = 163), and third AI (n = 83).

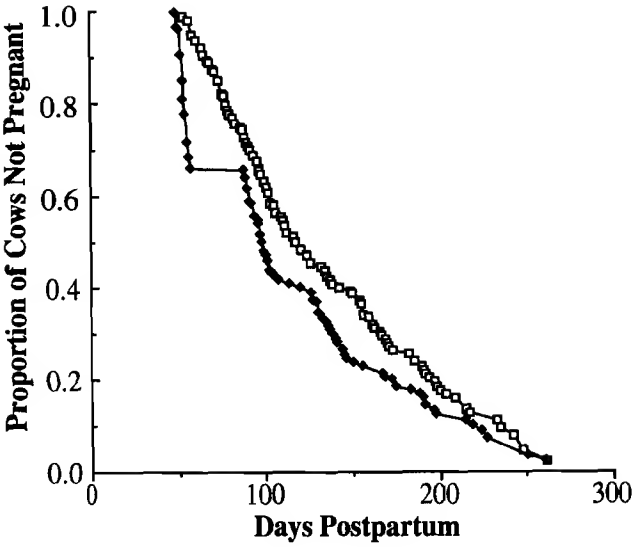


Figure 2. Survival curves for days to conception for lactating Holstein cows managed with standard reproductive strategies (□) versus timed AI after synchronization of ovulation (♦).

the efficiency of reproductive management of lactating dairy cows. As summarized in a recent literature review (10), no protocol has been available to dairy producers that "will consistently synchronize estrus with sufficient precision to permit high levels of success with fixed-time insemination." The protocol used in this study was not directed at synchronizing estrus, but at synchronizing the time of ovulation (17, 19). The management advantages of precisely controlled fixed-time AI were readily apparent from the results of this study.

One major advantage of the procedure for synchronization of ovulation is that producers can select a day for first AI rather than a voluntary waiting period (see Figure 1). Many herds use a short voluntary waiting period, such as 50 d, because of the concern that cows either will not conceive or will conceive too late in gestation (7, 15). The paradox, however, is that cows that become pregnant too early in lactation may be as costly as cows that conceive too late in lactation (4, 16). The optimal time of AI is not well defined and probably varies among cows. The advent of successful fixed-time AI makes it important for future research to elucidate a method that allows dairy producers to select rationally an optimal time for first AI.

Interestingly, the median intervals between first, second, and third AI were approximately 42 d for both control and treated cows. This interval was predesigned for cows with synchronized ovulation because those cows could only be reinseminated after pregnancy diagnosis on d 32 and resynchronization with the treatment protocol. Furthermore, although control cows could be reinseminated at each detected estrus, the intervals between first, second, and third AI were also approximately 40 d as has been previously observed with standard reproductive management (22). Because less than half of lactating cows become pregnant at each breeding (1, 19), it is important to identify nonpregnant cows as early in pregnancy as possible. Circumscribed estrus detection or milk progesterone assays at 18 to 25 d after AI may help to identify nonpregnant cows in some herds using synchronization of ovulation. Early pregnancy diagnosis becomes more critical when detection of estrus is not used for designation of nonpregnant cows. In most dairy herds, rectal palpation is used to determine pregnancy and would likely be performed at a later stage of gestation than the 32 d or less that can be accurately done with ultrasound. Thus, synchronization of ovulation and ultrasonography may provide a unique partnership in the management of reproduction in the future. The earlier diagnosis of pregnancy with ultrasound is likely to yield slightly greater pregnancy rates than that found with rectal palpation because of pregnancy losses that occur between 25 and 40 d of pregnancy.

The major limitation of previous protocols for timed AI has been the poor pregnancy rates per AI compared with AI to a detected estrus (10, 13, 23). In this study, synchronization of ovulation with GnRH and $PGF_{2\alpha}$ resulted in pregnancy rates for first, second, or third AI that were similar to those for standard reproductive management. In another study,

TABLE 2. Mean days postpartum when cows were culled from the herd in the treated and control groups.

| Treatment | 0 AI | | | ≥1 AI | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\bar{X}$ | SE | (no.) | $\bar{X}$ | SE | (no.) | $\bar{X}$ | SE | (no.) |
| Control | 71 | 7.1 | 23 | 195 | 12.5 | 12 | 114 | 12.0 | 35 |
| Treated | 52 | 0 | 3 | 131 | 12.6 | 22 | 121 | 12.2 | 25 |

Pursley et al. (19) found similar pregnancy rates per AI with timed AI after synchronization of ovulation compared with pregnancy rates with AI after an estrus synchronized with $PGF_{2\alpha}$. Interestingly, in this study, cows in the treated group received their first AI earlier in lactation than did control cows, at a stage of lactation when cows are generally considered to have lower fertility (4). In addition, all treated cows received AI, regardless of previous reproductive disorders, which makes it even more remarkable that pregnancy rates per AI were similar between the two groups. Cows with reproductive problems might have been less likely to be bred if assigned to the control group rather than the treated group because only 34% of control cows that were culled had received AI compared with 88% of the treated cows that were culled (Table 2).

Fertility in lactating dairy cows is clearly lower than is desired. Pregnancy rates of lactating dairy cows per AI have decreased from 66% in 1951, to about 50% in 1975, to about 40% currently (15, 19, 22). Some factors that limit fertility of lactating dairy cows include negative energy balance (21), toxic concentrations of urea and nitrogen (6), heat stress and other stresses (3, 12), and vitamin and mineral deficiencies (2). The physiologic effects of these factors have been difficult to assess because physiological and management factors may alter the accuracy and reliability of estrus detection as well as the pregnancy rate per AI. Obviously, synchronization of ovulation alone did not improve pregnancy rates per AI but did provide a research tool to allow manipulation of limiting factors in relation to precise time of ovulation. For example, Pursley et al. (18) evaluated the optimal time of AI in relation to ovulation, which previously could only be evaluated by studies using intensive detection of estrus (26, 27).

Days to conception have been used as one of the most economically important measures of reproduction in dairy cows. In this study, the median time to conception was reduced by 19 d for synchronized cows compared with cows managed using typical reproductive strategies. The median days to conception for cows in the control group (118 d) indicated good reproductive management because the mean number of days to conception for Wisconsin dairy herds that belong to Wisconsin DHIA was 132 d in December 1994 (D. A. McCullough, 1996, personal communication). Thus, this synchronization of ovulation protocol can reduce days to conception even in dairy herds with good reproductive management.

## CONCLUSIONS

This study examined whether reproduction of lactating dairy cows could be managed effectively without estrus detection by using a protocol that synchronized the time of ovulation. Treated cows had first AI at an earlier stage of lactation and had less variability in time to first AI than did control cows. Second and third AI were also approximately 30 d earlier for treated cows than for control cows, even though treated cows could not be inseminated to detected estruses until pregnancy diagnosis and resynchronization. Pregnancy rates per AI were similar for the two groups. The synchronization protocol reduced the median days to conception by 19 d. Thus, synchronization of ovulation provided an effective way to manage reproduction in lactating dairy cows by eliminating the need for estrus detection. This protocol should provide dairy producers with greater control over reproductive management than they have with conventional breeding practices.

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## GENETIC AND BREEDING

# Phenotypic Evaluation of Fertility Traits and Their Association with Milk Production of Italian Friesian Cattle[1]

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### ABSTRACT

The effect of environmental factors on first service conception rate, days from calving to first service, weeks from calving to conception, days from first to second service, and number of services per conception and the relationship of production with reproductive performance were estimated using 1.42 million records. The production trait was 305-d mature equivalent milk production. Season and age affected fertility: reproductive performance was lowest for lactations initiated during warm months (May to July) and decreased with parity. The association of production and fertility was antagonistic. High producing cows had 12 d more from calving to first service, .15 lower first service conception rate, .32 more services per conception, and 4.8 wk more from calving to conception than low producing cows in the same herd. The negative effect of production on fertility was reduced by superior herd management associated with high production. The difference in reproductive performance of cows in high and low producing herds represented only about 35% of the difference in the performance of high and low producing cows within herds for all fertility traits. The negative effect of production on fertility for interval between first and second service was totally compensated for by better management.

(**Key words**: Friesian cattle, fertility, production)

**Abbreviation key**: **CPC** = cow production class, **DBS** = days between first and second service, **DCFS** = days from calving to first service, **FSC** = first service conception rate, **HPC** = herd-year production class, **HS** = herd size, **305-d ME** = 305-d mature equivalent milk production, **SPC** = services per conception, **WCC** = weeks from calving to conception.

### INTRODUCTION

Milk production and reproductive performance are major factors affecting profitability of a dairy herd. Inadequate herd reproductive performance, manifested in prolonged calving intervals, increased forced culling, or both, can result in less milk and fewer calves per cow per year, less directional culling and therefore increased replacement cost, and, ultimately, lower net returns.

Using computer simulation, Oltenacu et al. (18) estimated an increase in net return per cow per year of $107 in herds with good reproductive performance (12.5-mo calving interval and 3% reproductive culling rate) relative to herds with poor reproductive performance (14-mo calving interval and 15% reproductive culling rate). For Dutch conditions, Dijkhuizen et al. (5) estimated total losses that were due to fertility problems at Dfl. 80/yr per cow. Pelissier (19) used a budgeting technique and estimated that the annual cost of infertility for the US dairy industry exceeded $116 per cow.

One prerequisite for effective management of reproduction is accurate quantitative knowledge of factors affecting reproductive performance, such as parity and season, and of the association of reproduction and other economi-

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cally important traits, such as milk production and length of productive life. Although infertility problems are of general interest, the parameters required for the management of infertility are specific to populations and need to be estimated periodically.

Many studies (3, 8, 11, 12, 14, 15, 17, 21) have shown that increased milk production is detrimental to the maintenance of high reproductive efficiency. When the association of production and fertility is estimated from field data, which often are clustered in herds, the potential confounding effect of the herd environment and the effect of the differential reproductive management of individual cows or groups of cows within a herd should be considered.

Intuitively, a favorable herd environment, which is conducive to both high milk production and reproductive performance, lessens the importance of the biologically antagonistic association of milk production and fertility. However, if high producing cows are given more breeding opportunities or if their first breeding is delayed relative to that of low producing cows in a herd (differential management), the association of production and fertility appears to be more antagonistic.

Some reproductive measures, such as days from calving to first service (**DCFS**), weeks from calving to conception (**WCC**), or number of services per conception (**SPC**) could be influenced by herd environment and by differential management within herds. Other measures, such as days between first and second services (**DBS**) or first service conception rate (**FSC**), are likely to be affected by herd environment only.

The first objective of this study was to evaluate the effects of parity and season of calving on fertility measures of Italian Friesian cattle. The second objective was to estimate the association of milk production and fertility measures. Our intent for the second objective was to separate the effect of stress of production from the effect of herd environment (which we consider to reflect herd management) in order to estimate the biological effect of milk production on fertility and the degree to which this association is changed by management. To achieve this objective, our premises were that 1) the difference in reproductive performance of cows in different cow production classes (**CPC**) within herds measures the biological effect of production on fertility without interference from management and 2) the difference in reproductive performance of cows in herds with different production levels measures the combined effect of production and herd environment (level of management) on fertility. The first premise holds true if the reproductive management of cows in different CPC within herds is relatively uniform; some indirect evidence is provided. The second premise implies that a positive association exists between the production level of a herd and its environment and that this association is mediated through the level of management in the herd. Superior herd management thus is conducive to high production and high reproductive performance. In the last decade, the trend in Italy has been toward larger herds, more modern technology, and generally better management. In this study, herd size (**HS**) was used as a secondary indicator of management, and the difference in reproductive performance of cows in small and large herds with similar production was used to provide additional evidence of the effect of herd environment on the association of production and fertility.

## MATERIALS AND METHODS

The data were collected by the Italian Breeders Association and consisted of production records with breeding information for Friesian cattle from 1966 to 1984. Five fertility traits and one production trait were considered. The fertility traits were FSC (1 = success; 0 = failure), SPC, WCC, DCFS, and DBS. The production trait was 305-d mature equivalent milk production (**305-d ME**) adjusted for age and for month of calving using the multiplicative adjustment factors developed by Bagnato et al. (2) for the National Breeders Association of the Italian Friesian Breed.

To calculate WCC, the service occurring within 282 ± 15 d prior to the date of subsequent calving was selected as date of pregnancy. If no service occurred within that range, insemination was assumed to be successful at 282 d prior to subsequent calving. To calculate DBS, the first two services were considered to be different if they were separated by at least 5 d.

Records meeting the following criteria were retained: 1) DCFS between 22 and 365 d, 2) WCC between 4 (22 to 28 d) and 54 wk, 3) 305-d ME >2000 kg, 4) lactation length >200 d, and 5) parity number ≤10. Only data for cows with consecutive records starting with first lactation were retained. The final data file consisted of 1.42 million records.

The breeding information available for the lactation record for each cow consisted of the dates of the past 6 inseminations and the type of service (natural or AI). Therefore, all records with >6 services were not considered when DCFS or DBS traits were analyzed.

The terminal record of each cow was not considered when the WCC trait was analyzed but was used for the analysis of all other fertility traits (for FSC and SPC traits, conception was assumed to occur at the last recorded service). A preliminary investigation was conducted in order to evaluate possible differences between a data file in which the terminal record was excluded for all fertility traits and a data file that included the terminal record. Agreement in the results using the two different data files provided justification for our approach. Means, standard deviations, and number of records for fertility and production traits are shown in Table 1.

Cows in each herd-year of calving were divided into terciles (low, medium, and high) that were defined using deviations of 305-d ME from herdmates (CPC). Italy was divided into seven milk-producing zones based on the prevailing dairy production system and the contribution to the total milk supply, as described by Aleandri et al. (1). Herd-year subclasses were ranked within each milk-producing zone and year using the 305-d ME average and classified in quartiles for herd-year production class (**HPC**); the first quartile represented the lowest producing herds. The number of calvings per calendar year was accurately reported and was available for all herd-year subclasses and was therefore used to represent HS, and each herd-year was classified as small, <21; medium, from 21 to 40; or large, >40.

TABLE 1. Means, standard deviations, and number of records for the fertility and productive traits.[1]

| Trait | | | Records |
|---|---|---|---|
| | $\bar{X}$ | SD | (no.) |
| FSC, % | .62 | .48 | 1,421,051 |
| SPC, no. | 1.63 | 1.05 | 1,421,051 |
| WCC, wk | 16.75 | 8.25 | 1,159,333 |
| DCFS, d | 87.05 | 36.21 | 1,414,492 |
| DBS, d | 50.82 | 37.09 | 565,494 |
| 305-d ME, kg | 6010 | 1428 | 1,421,051 |
| 305-d M, kg | 5472 | 1359 | 1,421,051 |

[1]FSC = First service conception rate, DBS = days between first and second service, DCFS = days from calving to first service, SPC = services per conception, WCC = weeks from calving to conception, 305-d M = actual 305-d milk production, and 305-d ME = 305-d mature equivalent milk production.

The following statistical model was used for the analysis:

$$Y_{ijklmn} = \mu + HY_i + P_j + M_k + I_l + CPC_m + e_{ijklmn}$$

where

$Y_{ijklmn}$ = cow lactation record for FSC, DBS, DCFS, SPC, WCC, and 305-d ME;
$\mu$ = overall mean;
$HY_i$ = fixed effect of herd-year of calving i;
$P_j$ = fixed effect of parity j;
$M_k$ = fixed effect of month of calving k;
$I_l$ = fixed effect of type of first service l (natural or AI);
$CPC_m$ = fixed effect of CPM m; and
$e_{ijklmn}$ = random residual effect.

The last level of each factor was set to zero. Least squares estimates and statistics for all effects were obtained after herd-year effects were absorbed. Backsolving was subsequently used to obtain least squares estimates for herd-year effects, which were used to calculate least squares means for the effect of HPC and the effect of HS by HPC. The standard errors of the least squares means for HPC and for HS by HPC were not calculated.

## RESULTS AND DISCUSSION

### Parity, Month of Calving, and Type of Insemination

All of the factors in the model had a significant effect for all traits analyzed except parity and month of calving for 305-d ME.

TABLE 2. Least squares estimates and their largest standard errors for the effect of parity on fertility traits[1] and actual 305-d mature equivalent milk production (305-d ME) expressed as differences from cows with parity = 10.

| Parity | FSC | SPC | WCC | DCFS | DBS | 305-d ME |
|---|---|---|---|---|---|---|
| | (%) | (no.) | (wk) | (d) | | (kg) |
| 1 | .12 | −.32 | −.4 | −1.0 | 5.9 | −352 |
| 2 | .10 | −.25 | −.6 | −2.6 | 4.2 | 141 |
| 3 | .09 | −.22 | −.6 | −2.7 | 3.6 | 420 |
| 4 | .07 | −.18 | −.5 | −2.4 | 3.5 | 514 |
| 5 | .06 | −.15 | −.4 | −2.4 | 3.0 | 489 |
| 6 | .05 | −.12 | −.3 | −2.1 | 2.4 | 387 |
| 7 | .04 | −.09 | −.5 | −2.4 | 2.1 | 250 |
| 8 | .04 | −.09 | −.6 | −1.7 | 1.1 | 110 |
| 9 | .02 | −.06 | −.1 | −.7 | 1.2 | 28 |
| SE | .02 | .04 | .04 | 1.2 | 1.9 | 18 |

[1]FSC = First service conception rate, DBS = days between first and second service, DCFS = days from calving to first service, SPC = services per conception, and WCC = weeks from calving to conception.

Least squares estimates for the effect of parity on the fertility measures are shown in Table 2. The general trend was a decrease in FSC and DBS and an increase in SPC as parity increased, but DCFS and WCC were unaffected. These results agree with those of many studies (6, 12, 13, 16, 20, 21) reporting that age is negatively correlated with fertility. The lack of an age effect on WCC was probably due, first, to the nature of our data (i.e., the terminal record for each cow was not considered when this trait was analyzed) and, second, to the small effect of age on DCFS.

Least squares estimates for the effect of calving month on reproductive measures are shown in Table 3. Many studies (6, 8, 12, 13, 20) report that reproductive performance of cows is affected by month of calving, but seasonal differences differ in magnitude and direction for different cattle populations, coun-

TABLE 3. Least squares estimates and largest standard errors for the effect of month of calving and of type of service on fertility traits,[1] expressed as differences from cows calving during December and from cows inseminated by natural service, respectively.

| Item | FSC | SPC | WCC | DCFS | DBS |
|---|---|---|---|---|---|
| | (%) | (no.) | (wk) | (d) | |
| Month of calving | | | | | |
| Jan | .01 | 0 | .4 | −0 | 2.7 |
| Feb | 0 | .01 | .4 | −1.3 | 3.9 |
| Mar | .01 | 0 | .6 | −.2 | 6.0 |
| Apr | 0 | .01 | 1.0 | 2.0 | 7.1 |
| May | −.01 | .03 | 1.4 | 4.4 | 6.6 |
| Jun | −.03 | .06 | 1.2 | 3.3 | 4.1 |
| Jul | −.01 | .03 | .8 | 3.0 | 3.0 |
| Aug | −.01 | .02 | .6 | 2.2 | 1.6 |
| Sep | −.01 | .02 | .5 | 2.1 | .8 |
| Oct | −.01 | .02 | .3 | 1.4 | .2 |
| Nov | −.01 | .02 | .2 | .7 | −.4 |
| SE | .002 | .004 | .04 | .15 | .25 |
| Type of service[2] | | | | | |
| AI | −.04 | . . . | . . . | −5.9 | . . . |
| SE | .001 | . . . | . . . | .09 | . . . |

[1]FSC = First service conception rate, DBS = days between first and second service, DCFS = days from calving to first service, SPC = services per conception, and WCC = weeks from calving to conception.

[2]Shown only for FSC and DCFS.

tries, or regions. In the present study, cows calving in April, May, June, and July had lower reproductive performance, reflected in lower FSC, higher SPC, and more DCFS, DBS, and WCC. These cows had their breeding period during the warm months of June through September, and heat stress may have been responsible for lower reproductive performance. Temperatures in different regions of Italy vary from a maximum of 33 to 44°C during warm months to a minimum of 2 to −15°C during winter. The increase of 5 to 10 d for WCC for these cows resulted from a 2- to 4-d increase in DCFS and a 3- to 7-d increase in DBS. Faust et al. (7), using several research herds in North Carolina, reported seasonal differences of similar magnitude in reproductive performance. Gwazdauskas et al. (9, 10) also reported lower fertility associated with warm summer months.

Least squares estimates for the effect of type of insemination (AI vs. natural service) on FSC and DCFS are shown in Table 3. The effect is shown for FSC and DCFS only because the information for type of insemination referred to first service only. Higher FSC and a 6-d increase in DCFS occurred with natural service.

#### Cow and Herd Production Level

Least squares estimates for the effect of CPC and of HPC on the reproductive performance measures, expressed relative to the class of high producing cows and the class of high producing herds, are shown in Table 4. Our assumption was that the effect of CPC represents a measure of the effect of stress of production on fertility without interference from management, whereas the effect of HPC represents a combination of the effect of production and the effect of herd environment on fertility.

The validity of this assumption depends on the absence of differential reproductive management of cows in different CPC.

A separate analysis was performed for actual 305-d milk production as a production trait, and the least squares estimates for the effect of parity are in Table 2. These results show that actual 305-d milk production for cows in parities 2, 3, and 4 was 493, 772, and 866 kg greater than for first parity cows, but the differences in DCFS were only 1.6, 1.7, and 1.4 d, respectively; therefore, first breeding policy was not influenced by the cow's production. A similar conclusion was reached by Berger et al. (3), working with US data. The differences in WCC with parity, shown in Table 2, were also small. Because DCFS and WCC are the two reproductive traits that are most likely to be affected by differential reproductive management, these results suggest that differential reproductive management was not a common practice in these data and support the underlying assumption for the in-

TABLE 4. Least squares estimates and largest standard errors for the effect of cow production class (CPC)[1] and differences in least squares means[2] for the effect of herd-year production class (HPC) on fertility traits[3] and on 305-d mature equivalent milk production (305-d ME).

| Production class | FSC | SPC | WCC | DCFS | DBS | 305-d ME |
|---|---|---|---|---|---|---|
| CPC | (%) | (no.) | (wk) | ——— (d) ——— | | (kg) |
| Low | .15 | −.32 | −4.8 | −11.9 | −6.3 | −2219 |
| Medium | .09 | −.18 | −2.6 | −6.1 | −2.7 | −1062 |
| SE | 0 | .002 | .02 | .1 | .12 | .1 |
| HPC | | | | | | |
| Low | .08 | −.16 | −1.9 | −5.0 | 2.4 | −2574 |
| Average | .04 | −.09 | −1.3 | −3.6 | 2.0 | −1643 |
| Good | .02 | −.04 | −.7 | −2.4 | 1.5 | −992 |

[1]Expressed as differences from high producing cows.

[2]Expressed as differences from herds with high production. Standard errors not calculated.

[3]FSC = First service conception rate, DBS = days between first and second service, DCFS = days from calving to first service, SPC = services per conception, and WCC = weeks from calving to conception.

terpretation of the effect of CPC and HPC on fertility.

Least squares estimates for the effect of CPC on fertility measures (Table 4) indicate that production had a negative effect on fertility. Cows in the high producing tercile had .15 lower FSC, required .32 more SPC, had 4.8 wk longer WCC, had 12 d longer DCFS, and had 6.3 d longer DBS than cows in the low producing tercile. This antagonistic association is well documented by Berger et al. (3), Hillers et al. (12), Laben et al. (15), Olds et al. (17), and Spalding et al. (21), who report that high producing cows had lower FSC, more SPC, more DCFS, and longer WCC than low producing cows.

To determine which fertility trait is more affected by production, the least squares estimates of the differences in reproductive performance of cows in different production groups (Table 4) can be expressed as percentage of the population averages for the respective fertility traits (Table 1). The changes in fertility traits for high relative to low producing cows, in increasing order, were 12, 16, 20, −24, and 29% for DBS, DCFS, SPC, FSC, and WCC, respectively.

The effect of production on FSC, DCFS, and DBS estimated from our study were compared (Table 5) with those of Spalding et al. (21), who used a similar statistical model to estimate these effects for Holstein cattle in DHI herds in New York. To facilitate the comparison, the differences between cows in different CPC for fertility traits were expressed relative to the difference in production between CPC. Spalding et al. (21) estimated the effect of production for increments of 907 kg in actual 305-d milk production, which was calculated to be equivalent to the effects of 1000 kg of deviation in 305-d ME in our study. For increases of 1000 and 2000 kg in CPC, FSC decreased by .08 and .14 in our study and by .06 and .11 in the study of Spalding et al. (21); however, when FSC was expressed as percentages of the average for the two populations (.62 and .50, respectively), the decreases in FSC were similar in both studies.

For increases of 1000 and 2000 kg for production of CPC, DBS increased by 2.5 and 5.6 d in our study and by 3.6 and 7.5 d in the study of Spalding et al. (21). When DBS was expressed as percentages of the average in the two populations (50 and 41 d, respectively), these changes were twice as large for New York Holsteins as in the Italian Friesian population. The effect of production on DCFS was also greater in our study than in the study of Spalding et al. (21); DCFS increased 5.7 and 10.8 d in our study and 3.8 and 8.3 d in the study of Spalding et al. (21) for increases of 1000 and 2000 kg in production of CPC, respectively.

Differences on least squares means for the effect of HPC on fertility measures (Table 4) indicate that the combined effect of production

TABLE 5. The effect of cow's milk deviation from herdmates as least squares estimates[1] and percentage of population average[2] on first service conception rate (FSC), days to first service (DCFS), and days between first and second service (DBS) of Italian Friesians and of New York Holstein.[3]

| | | Italian Friesians | | New York Holsteins | |
|---|---|---|---|---|---|
| Fertility trait | Cow's milk deviation | Least squares estimates | Population mean | Least squares estimates | Population mean |
| | (kg) | | (%) | | (%) |
| FSC, % | +1000 | −.085 | 13.7 | −.063 | 12.6 |
| | +2000 | −.139 | 22.4 | −.111 | 22.2 |
| DCFS, d | +1000 | 5.7 | 6.5 | 3.8 | 4.4 |
| | +2000 | 10.8 | 12.4 | 8.3 | 9.5 |
| DBS, d | +1000 | 2.5 | 4.9 | 3.6 | 8.8 |
| | +2000 | 5.6 | 11.0 | 7.5 | 18.3 |

[1]Least squares estimates of the effect expressed as deviation from the reproductive performance of low producing cows in the herd.

[2]Least squares estimates expressed as a percentage of the population average for the respective reproductive measure.

[3]As reported by Spalding et al. (21).

and herd environment on fertility was antagonistic but smaller than the effect of production alone. Using a similar strategy in which herds were stratified by production, Butler and Smith (4) and Laben et al. (15) reported comparable differences in fertility traits.

To assess the degree to which the antagonistic association of production and fertility is tempered by the more favorable herd environment of better producing herds, the effect of CPC on fertility was compared with the effect of HPC. To facilitate this comparison, the effects of CPC and HPC on fertility traits were expressed per 500-kg increments of 305-d ME; the values, calculated by interpolation using the values in Table 4, are presented in Table 6. The results in Table 6 show that the degree to which superior herd management, which is associated with higher herd production, reduces the negative effect of production on fertility varies with the fertility trait and with the production deviation considered. For example, the negative effect of an increase of 2000 kg in HPC on FSC, SPC, WCC, and DCFS was equal to 64, 59, 66, and 62% of the effect of the same increase in production of CPC, respectively. For DBS, the negative effect on production was totally compensated for by herd management, and the direction of the association between production and DBS was reversed. The values in Table 6 also show that the degree to which superior management reduces the negative effect of production on fertility decreases as the production deviation for FSC trait increases, but remains relatively constant for the other fertility traits over the range of production increments studied.

### HS

Differences in least squares means for the effect of HS on reproductive performance are shown in Table 7. The results indicate an improvement in reproductive performance with HS except for FSC, which decreases, and SPC, which increases. Similar results were reported by Spalding et al. (21).

Within each HPC, herd production increased with HS, but the increase was largest among herds in the first quartile (the 25% lowest producing herds). In that group, the difference between large and small herds was 812 kg in 305-d ME. For second, third, and fourth quartiles of HPC, the differences in production between small and large herds decreased to 562, 492, and 457 kg, respectively. The effect of HS within HPC on reproductive traits followed the same trend as the effect of herd production, providing additional evidence that better management could

TABLE 6. The effect of increased production[1] and the combined effect of increased production and superior management[2] on fertility traits.[3]

| Milk production deviation | FSC | SPC | WCC | DCFS | DBS |
|---|---|---|---|---|---|
| (kg) | (%) | (no.) | (wk) | (d) | |
| Production effect | | | | | |
| +500 | −.04 | .08 | 1.2 | 2.9 | 1.3 |
| +1000 | −.08 | .17 | 2.4 | 5.7 | 2.5 |
| +1500 | −.11 | .23 | 3.4 | 8.3 | 4.1 |
| +2000 | −.14 | .29 | 4.4 | 10.8 | 5.6 |
| +2500 | −.16 | .35 | 5.3 | 13.3 | 7.2 |
| Combined effect | | | | | |
| +500 | −.01 | .02 | .4 | 1.2 | −.8 |
| +1000 | −.02 | .04 | .8 | 2.4 | −1.5 |
| +1500 | −.03 | .08 | 1.1 | 3.3 | −1.9 |
| +2000 | −.05 | .12 | 1.5 | 4.1 | −2.1 |
| +2500 | −.08 | .15 | 1.8 | 4.9 | −2.4 |

[1]The production effects were calculated by interpolation using values for cow production class (CPC).

[2]The combined effects were calculated by interpolation using values for herd-year production class (HPC).

[3]FSC = First service conception rate, DBS = days between first and second service, DCFS = days from calving to first service, SPC = services per conception, and WCC = weeks from calving to conception.

TABLE 7. Differences in least squares means[1] for the effect of herd size (HS) by herd production class (HPC) on fertility traits,[2] expressed as differences from large herds with high production.

| HPC | HS | FSC | SPC | WCC | DCFS | DBS | 305-d ME[3] |
|---|---|---|---|---|---|---|---|
| | | (%) | (no.) | (wk) | (d) | | (kg) |
| Low | Small | .19 | −.43 | −1.9 | −.7 | 4.5 | −2851 |
| | Medium | .08 | −.22 | −1.2 | −2.0 | 4.3 | −2205 |
| | Large | .06 | −.15 | −1.2 | −3.0 | 4.4 | −2039 |
| Average | Small | .16 | −.37 | −1.0 | 2.0 | 4.3 | −1982 |
| | Medium | .07 | −.19 | −1.1 | −1.6 | 3.4 | −1577 |
| | Large | .03 | −.08 | −.7 | −2.6 | 2.2 | −1420 |
| Good | Small | .14 | −.34 | −.5 | 4.2 | 4.9 | −1395 |
| | Medium | .06 | −.16 | −.7 | −.8 | 2.4 | −1080 |
| | Large | .01 | −.02 | −.4 | −1.7 | .4 | −903 |
| High | Small | .12 | −.30 | .5 | 7.7 | 4.1 | −457 |
| | Medium | .05 | −.15 | −.2 | 1.6 | 1.7 | −199 |

[1]Effects estimated by backsolving (standard errors not available).

[2]FSC = First service conception rate, DBS = days between first and second service, DCFS = days from calving to first service, SPC = services per conception, and WCC = weeks from calving to conception.

[3]305-d Mature equivalent milk production.

decrease the antagonism between production and fertility. The effect of HS on FSC and SPC was essentially the same for each quartile of HPC, but, for DCFS and DBS, the effect of HS increased with HPC. For example, the difference in FSC between large and small herds was −.13 across all HPC, but the differences in DCFS were −2.3, −4.6, −5.9, and −7.7 d for first, second, third, and fourth quartiles of HPC, respectively.

Both HPC and HS are indirect indicators of the herd level of management. Although the differences in production between small and large herds are smaller, the difference in the level of management between small and large herds may be greater in the high producing group than in the low producing group. A larger difference in management level could explain the greater effect of HS within HPC on the fertility traits that are more sensitive to management (DCFS and DBS) compared with less sensitive traits (FSC and SPC).

## CONCLUSIONS

Parity and season of calving affected fertility of Italian Friesian cattle. Reproductive performance decreased as parity increased and was lower for lactations initiated during warm summer months. Different measures of production were used in this study to separate the effect of stress of production from the effect of herd environment (level of management) on fertility. The association of production and fertility was antagonistic. The superior management prevailing in high producing herds substantially reduced the negative effect of production stress on fertility, but the degree to which this antagonism was reduced decreased as production increased. The positive effect of superior management was similar for FSC, SPC, DCFS, and WCC, for which the negative effect of production was reduced by about 65%, but was greater for DBS, for which the direction of the association was reversed.

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# Use of Prostaglandin $F_{2\alpha}$ as a Postpartum Reproductive Management Tool for Lactating Dairy Cows

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## ABSTRACT

This study compared three programs for reproductive management of the postpartum period for reproductive performance and net economic benefit within three dairy herds (n = 1624 cows). Cows on one program received $PGF_{\alpha}$ injection at 25 to 32 d postpartum for reproductive therapy, and cows on a second program received additional $PGF_{2\alpha}$ at 39 to 46 d postpartum for synchronization of estrus. These programs were compared with a postpartum program of rectal palpation based on veterinary intervention. Survival analysis indicated that cows receiving $PGF_{2\alpha}$ for reproductive therapy and synchronization of estrus had an 11% higher rate of first AI and 10% higher rate of pregnancy than did cows receiving the rectal palpation. No differences existed between the cows receiving rectal palpation and those receiving the $PGF_{2\alpha}$. Because overall conception rates and conception rates at first AI did not differ among programs, the improved reproductive performance of cows receiving $PGF_{2\alpha}$ for both therapy and synchronization may be attributed to greater synchronization of estrus, which resulted in improved estrus detection. A partial budget indicated that the $PGF_{2\alpha}$ programs were less expensive than the rectal palpation program. When $PGF_{2\alpha}$ was used for postpartum reproductive therapy and synchronization of estrus, reproductive performance and net economic benefit were increased compared with those of the other programs. (**Key words**: prostaglandin $F_{2\alpha}$, reproductive efficiency, reproductive programs)

**Abbreviation key**: **PG** = postpartum therapeutic $PGF_{2\alpha}$, **RP** = rectal palpation, **SI** = $PGF_{2\alpha}$ at a scheduled interval.

## INTRODUCTION

Reproductive efficiency is often a limiting factor of dairy herd productivity and profitability (13, 21, 24, 25). Profits from a reproductive program are maximized when a majority of cows exhibit optimal reproductive performance (21, 25). Most economic models show an 11- to 13-mo calving interval as optimal for dairy cows (21, 24, 25). Rounsaville et al. (29) determined that detection of estrus and rate of conception were the major factors affecting reproductive performance. Because nearly half of the estrous periods in normally cycling cattle may be not detected (29), poor detection of estrus in herds using AI contribute to increased days open. Several studies (2, 29) have linked poor estrus detection to lengthened calving intervals. Therefore, economic losses associated with lengthened calving intervals often go undetected by dairy producers (25). Ranges of $.25 to $4.68/d open per cow beyond 85 d postpartum have been used to quantify costs of reproductive inefficiency (9).

Historically, postpartum reproductive programs have been based on routine rectal palpation (**RP**) of individual cows (11). Veterinary intervention may be based on routine RP examinations. Presently, the effectiveness of these traditional programs is in question. One alternative is the use of $PGF_{2\alpha}$ or its analogs

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as a tool for postpartum reproductive therapy (PG) and estrus detection or replacement of routine RP with programs based on the administration of $PGF_{2\alpha}$ at scheduled intervals (SI).

Regulation of estrus in cows has been achieved through the use of $PGF_{2\alpha}$ (8, 10, 19, 28, 30). Cows with a corpus luteum are expected to be in estrus within 120 h after treatment with $PGF_{2\alpha}$ (30). Although the licensed use of $PGF_{2\alpha}$ concerns synchronization of estrus, estrus induced by $PGF_{2\alpha}$ injected at 24 d postpartum resulted in a cleansing effect on the uterine environment and increased conception rates (7). Days open were fewer for cows treated with $PGF_{2\alpha}$ (30). Sequential injections of $PGF_{2\alpha}$ resulted in synchronization of estrus (8, 10, 19, 28, 30). Because RP is not sensitive for correctly identifying functional corpus lutea (18, 22, 26), improvements in detection of estrus and conception may result from SI to all open cows. Moreover, management of reproductive health would be based on the health of the whole herd rather than on the health of individual cows. However, a few studies (10, 19, 28) prospectively compare the effects of administration of $PGF_{2\alpha}$ to all open cows with those of a concurrent group involving routine RP. One uncontrolled field study (12) reported improved reproductive efficiency of open cows receiving SI. During the trial, days open decreased from 129 to 106 d (12). Controlled research (19, 30) suggested that an SI program might reduce days open and associated costs.

Thus, in this study, two reproductive management scenarios were developed, and each was compared with a program based on routine RP and intrauterine therapies. One protocol incorporated the following concepts: 1) RP is not sensitive for correctly identifying functional corpus lutea (18, 22, 26), 2) controlled studies indicate that the use of antibiotic or disinfectant uterine infusions may not be beneficial (23, 29), and 3) estrus induced by therapeutic use of PG may cleanse the uterine environment and increase fertility (7). Therefore, this protocol was designed to compare the therapeutic effects of $PG_{2\alpha}$ (Lutalyse™; Upjohn, Kalamazoo, MI) with the effects of postpartum therapies associated with routine RP. A second protocol incorporated the following concepts in addition to those of the other protocol: 1) SI may result in synchronization of estrus and improved reproductive efficiency (10, 19, 30), and 2) a higher percentage of estrus and conception, resulting in improved reproductive performance, was achieved when $PGF_{2\alpha}$ was administered at a 14-d interval rather than at an 11-d interval (10, 28). Thus, the second protocol was designed to compare the effects of PG combined with the use of $PGF_{2\alpha}$ to synchronize estrus with the effects of a program based on routine RP and intrauterine therapies.

The objective of this study was to compare, for herds using $PGF_{2\alpha}$ at the onset of the breeding period, three additional interventions: $PGF_{2\alpha}$ at 25 to 32 d postpartum, $PGF_{2\alpha}$ at both 25 to 32 d and at 39 to 46 d postpartum, and routine RP with no $PGF_{2\alpha}$ other than the use during the breeding period. The outcomes considered for the comparisons were days to first AI, days open, and net economic benefit. We hypothesized that the use of PG, replacing reproductive programs based on routine RP and intrauterine therapies, would result in a cost effective reduction in days open and improved reproductive efficiency. Subsequently, we postulated that this cost effective reduction in days open and improved reproductive efficiency would be further augmented through the combined use of PG for estrus detection, replacing routine RP with programs based on the administration of SI.

## MATERIALS AND METHODS

### Herd Selection

Before the inception of this study, specific criteria for herd participation were established so that the length of the study would not exceed 2 yr and participant herds would represent high management. Three cooperating clinicians from the Perry Veterinary Clinic (Perry, NY) were asked to identify herds that had >300 lactating Holstein cows that averaged >8636 kg of milk and <150 d open in the previous year. Table 1 contains baseline means for selected variables of the three Holstein herds. Participating herds were registered in the official Northeast DHI testing program (Ithaca, NY), and on-farm computerized records were maintained.

TABLE 1. Previous 12-mo means for reproductive performance of participating herds at initiation of study.

| Term | Herd 1 (n = 345) | | Herd 2 (n = 887) | | Herd 3 (n = 439) | |
|---|---|---|---|---|---|---|
| | $\bar{X}$ | SD | $\bar{X}$ | SD | $\bar{X}$ | SD |
| Days to first AI | 82 | 22 | 87 | 29 | 81 | 25 |
| Days open | 149 | 114 | 117 | 71 | 131 | 67 |
| Overall conception rate, % | 34 | 2.6 | 59 | 1.7 | 50 | 2.4 |
| Percentage of cows culled | 35 | 2.5 | 20 | 1.3 | 25 | 2.1 |
| 305-d ME[1] Milk, kg | 11,236 | 1889 | 10,026 | 1330 | 11,069 | 1040 |

[1]Mature equivalent.

### Cow Assignment

At parturition, cows (n = 1624) were assigned randomly within herd and by parity group (*primiparous or multiparous*) to one of three reproductive management protocols. All cows in participating herds had equal opportunity for inclusion. However, cows defined by the producer as ineligible for breeding were excluded (herd 1, 8 out of 345; herd 2, 29 out of 887; and herd 3, 10 out of 439). Criteria for not rebreeding cows were herd-specific and generally based on production during a previous lactation, postpartum disorders, or breeding difficulties in a previous lactation. The study began March 10, 1992 and concluded October 4, 1993; cows were assigned from March 10, 1992 to July 1, 1993.

Before study initiation, a 10-d difference in days open was hypothesized to be essential to justify economically a particular protocol for reproductive management. Calculations of sample size indicated that a minimum of 415 cows per treatment was needed to detect a 10-d difference in days open, allowing compensation for losses (estimated as 10% of total enrollment). Sample size was determined using the parameters $\alpha = \beta = .05$ and SD = 40 d open, and a detectable difference of 10 d open (17).

### Definition of Postpartum Reproductive Treatments

Before this study, in all three participating herds, a reproductive program based on routine RP and intrauterine therapy was followed, and $PGF_{2\alpha}$ was not used routinely. Experimental reproductive management protocols differed during the postpartum period by treatment group. To compare postpartum treatment effects, all cows received equivalent management programs during breeding and pregnancy. In addition, to reduce the potential for producer bias in management, detection of estrus, and veterinary treatment, no leg bands or other visual identification markers were used to identify cows by treatment. All data on treatment identification, collected monthly by the investigator, were recorded and stored on-farm using Dairy COMP 305® (Valley Agricultural Software, Tulare, CA).

*RP Treatment.* Cows receiving the RP treatment served as controls and followed a traditional reproductive program based on RP and associated therapies (e.g., intrauterine infusions and hormone therapy). All cows were palpated by a veterinarian at approximately 30 d (postpartum exam) and again at 50 d postpartum (prior to breeding exam). No $PGF_{2\alpha}$ was administered before the beginning of the breeding period. During the breeding period, $PGF_{2\alpha}$ was used at the discretion of the producer and veterinarian.

*PG Treatment.* Cows were administered a single injection of $PGF_{2\alpha}$ at 25 to 32 d postpartum in the PG treatment. To facilitate direct comparison of treatment effects between PG and RP, RP and associated therapies were prohibited in this treatment prior to the $PGF_{2\alpha}$ injection at 53 to 60 d postpartum.

*SI Treatment.* For the SI treatment, cows were injected with $PGF_{2\alpha}$ at 25 to 32 d and again 14 d after the initial injection (39 to 46 d postpartum). Cows observed to be in estrus following SI treatment were not bred. To facilitate direct comparison of treatment effects

between SI and RP, routine RP and associated therapies were prohibited in SI.

To ensure an equivalent initial breeding period for each treatment and to differentiate the breeding period from the treatment effect, all cows were injected with $PGF_{2\alpha}$ at 53 to 60 d postpartum. Cows could not be inseminated until after they had received at least one injection of $PGF_{2\alpha}$. Therefore, a voluntary waiting period of 56 d was established for all herds. No estrus detection aids were used, and cows were artificially inseminated if they were observed to be in estrus or were retreated with $PGF_{2\alpha}$ 14 d after the initial injection if they were not bred. Estrus detection on participating farms consisted of daily visual observation at routine intervals. Any cow not observed to be in estrus by 70 d postpartum received veterinary intervention and returned to the biweekly schedule of $PGF_{2\alpha}$ injections at the producer's discretion.

All inseminated cows were examined for pregnancy by RP at 35 to 40 d post AI. Open cows were administered $PGF_{2\alpha}$ at 14-d intervals until they were observed to be in estrus and then were reinseminated.

#### Data Analyses

Because pregnancy was the event of interest for analyses of days open and first AI was the event of interest for analyses of days to first AI, cows that had not reached the breeding period (53 to 60 d postpartum) were excluded from analyses.

Analyses were performed using BMDP statistical software (4). The predictor variable was treatment. Cows were to be assigned to treatment randomly, within herd and by parity group (primiparous or multiparous). However, total cow enrollment during the period of study (RP = 533, PG = 498, and SI = 510) was inconsistent with expected differences across treatments. Therefore, parity group was considered to be a potentially confounding variable, and adjustments in the analyses were made for parity in addition to season of parturition and herd. Seasonal categories were March to May, June to August, September to November, and December to February.

#### Survival Analysis: Days Open

The effects of treatment on days open was quantified using survival analysis. Survival analysis is a regression technique for data analysis in which the outcome variable is timed to an event (1, 3, 15, 16). The outcome variable for this analysis was a postpartum interval, days open, defined as the time between parturition and conception. Use of survival analysis for analysis of reproductive data is advantageous because the probability of an event (e.g., pregnancy) is calculated for consecutive days postpartum, enabling simultaneous analysis of censored and uncensored data (1, 3, 15). Censored data are contributed by those cows (open cows) not experiencing the event of interest (pregnancy). In this analysis, data for cows that were culled during days open after the end of the voluntary waiting period and data for cows that were still not pregnant by the end of the study were considered to be censored. Data for an open cow with DIM greater than or equal to the voluntary waiting period was also considered to be censored. For censored records of cows that were inseminated, days open was calculated as days from parturition to last date of AI (unconfirmed pregnancy) or last date of data collection (confirmed open). For censored data for cows that were not inseminated, days open were calculated as days from parturition to the date of culling or the last date of data collection.

Data were analyzed using the following Cox proportional hazards regression model:

$$h_{ij}(t) = h_{0j}(t)\exp\{\text{treatment} + \text{lactation} + \text{season}\}.$$

This analysis was stratified by herd because pregnancy rates (number of pregnancies per time) varied between herds. The probability of cow i in herd j becoming pregnant at t days after parturition was described by $h_{ij}(t)$, the hazard function. When all covariants were equal to 0, the unknown baseline probability of pregnancy was characterized by $h_{0j}(t)$, the baseline hazard function. For each independent variable in the model, a hazard ratio (analogous to a relative risk) was estimated. Hazard ratios, which estimate the rate of pregnancy with a base, were calculated raising exponentially 2.718 to the beta power. The pregnancy rate used in survival analysis is a true rate because survival analysis estimates the number of pregnancies per unit of time (1, 3, 19, 20). The difference between treatments that were

described by the hazard ratio was assessed by independently plotting Kaplan Meier estimators for the RP and SI treatments to obtain median days open. All tests of statistical significance ($P < .05$; two-sided) in the regression analysis were performed using the likelihood ratio test. A partial likelihood method was used to estimate the parameters of the model. In addition to main effects, all second-order interactions were estimated and were nonsignificant.

#### Survival Analysis: Days to First AI

The effects of treatment on days to first AI were quantified using survival analysis. The outcome variable for this analysis was a postpartum interval, days to first AI, defined as the time between parturition and first AI. In this analysis, data for cows that were culled after the end of the voluntary waiting period but before they were inseminated and cows that were still not inseminated by the end of the study were considered to be censored. Essentially, data for noninseminated cows with DIM greater than or equal to the voluntary waiting period were considered to be censored for this analysis. For data on censored cows, days to first AI were calculated as days from parturition to the date of culling or the last date of data collection. Data analysis was performed exactly as for days open, except that the outcome variable was first AI rather than pregnancy, and the maximum censoring time was the maximum observed days to first AI, 163 d.

#### Additional Analyses

Chi-squares with a Type 1 error of .05 were used for pooled data across herds; descriptive statistics were compared for first AI conception rates, culling rates, and the proportion of pregnancies among treatments. The cumulative incidence of postpartum disorders (including displaced abomasum, ketosis, metritis, milk fever, ovarian cysts, pyometra, retained placenta) was summarized and compared by inspection across treatments. Finally, a partial budget was used to evaluate the net benefits of the three treatments. Costs reflected fees charged by the Perry Veterinary Clinic from March 1992 to October 1993. Costs included in the analysis were rectal palpation fees ($2.25 per palpation), drug costs of $PGF_{2\alpha}$ ($2.25 per dose), and postpartum therapy ($10.88 per treated cow). The sensitivity analyses were done only for the cost of $PGF_{2\alpha}$ because $PGF_{2\alpha}$ was the focus of the study. The therapeutic costs were calculated by the actual incidence of health events and associated costs in the three herds.

## RESULTS

#### Days Open

The analysis was based on 472 RP cows on the treatment, 443 on PG treatment, and 461 on SI treatment. Of the data included in the analysis, records were censored for 93, 97, and 79 cows on the RP, PG, and SI treatments, respectively. The SI treatment was the only significant variable in the final Cox proportional hazards regression model (Table 2). No difference existed in hazard ratios between PG and RP treatments (Table 3). The hazard ratio for SI treatment was 1.10 when all other covariables were controlled. Therefore, after parity group (1 or >1) and season of parturition were controlled, the SI treatment resulted in a hazard ratio of 1.10, which indicates that cows on the SI treatment had a 10% higher pregnancy rate than did cows on the RP treatment, which may be a result of greater synchronization of estrus postpartum.

Sixty-one, 55, and 49 cows receiving RP, PG, and SI treatments, respectively, were culled prior to breeding, and their records were not included in the analysis. The exclusion of records for cows that were culled before breeding prompts the question of confounding. Confounding would be a concern if culling rate were related to treatment and outcome. However, the data indicate that postpartum treatment likely did not affect culling rate (RP = 11%, PG = 11%, and SI = 10%). Because treatment and culling rate are probably not related, no potential exists for confounding when these data are excluded from analyses. Finally, records for all cows with DIM ≥56 d postpartum were included in the analysis: 472 RP, 443 PG, and 461 SI records satisfied this criterion for inclusion.

Kaplan Meier estimators were independently plotted for SI and RP treatments to illustrate the cumulative pregnancy rates of

TABLE 2. Cox proportional hazard multiple regression model for days open.[1]

| Term | Coefficient[2] | SE | Wald[3] | LRCS[4] |
|---|---|---|---|---|
| Lactation[5] | −.01 | .07 | .86 | . . . |
| Season[6] | | | | |
| 1 | −.01 | .12 | .98 | . . . |
| 2 | .14 | .09 | .14 | . . . |
| 3 | .06 | .09 | .53 | . . . |
| Treatment[7] | | | | |
| 1 | −.08 | .06 | .18 | .19 |
| 2 | .09 | .04 | .03 | .03 |

[1]Analysis was stratified by herd.

[2]The coefficients for the categorical variables represent the $\log_e$ of the ratio of hazard functions for different levels compared with the base level.

[3]Wald statistic test is based upon the asymptotic normality of the maximum likelihood estimates.

[4]Likelihood ratio chi-square statistic for treatment.

[5]0 = 1, 1 = >1.

[6]0 = December to February, 1 = March to May, 2 = June to August, and 3 = September to November.

[7]0 = Rectal palpation (n = 472); 1 = postpartum therapeutic prostaglandin (n = 443), and 2 = scheduled interval prostaglandin (n = 461).

cows on these treatments (Figure 1). This technique determined median days open for SI treatment at 107 d and for RP treatment at 113 d (Figure 1). Because hazard ratios of PG and RP treatments were not statistically different ($P > .05$), the cumulative pregnancy rate of cows on the PG treatment was not plotted.

The impact of any violations of independence between censoring and the occurrence of the event was assessed by substituting extreme values for censored observations. Once the model was estimated, then the data were altered to the time of censoring had the event occurred (i.e., confirmed pregnant). Another time, all censoring times were set to be equal to the maximum time value (339 d open) observed in the data, and the model was again estimated. The parameter estimates from the standard analysis and the two extreme analyses were similar, which satisfied the assumption of independence (1, 3, 14, 15).

The assumption of the Cox proportional hazards model that the effects of treatment were independent of time (the proportional hazards assumption) also was tested. Each independent variable was stratified, and logarithmic transformations of the cumulative hazard function for each stratum were plotted. The survival curves were parallel (by inspection) and thus satisfied the assumption of proportional hazards (1, 3, 14, 15).

**Days to First AI**

The analysis was based on 472 cows on RP treatment, 443 on PG, and 461 on SI. Of the cows included in the analysis, 41 on RP, 41 on PG, and 29 on SI were censored. The SI treatment was the only significant variable in

TABLE 3. Hazard ratios and 95% confidence intervals (CI) for days open model.[1]

| Term | Hazard ratio[2] | 95% CI |
|---|---|---|
| Lactation[3] | .99 | .5 to 1.1 |
| Season[4] | | |
| 1 | .99 | .6 to 1.5 |
| 2 | 1.16 | .9 to 1.7 |
| 3 | 1.06 | .6 to 1.4 |
| Treatment[5] | | |
| 1 | .93 | .7 to 1.2 |
| 2 | 1.10 | 1.03 to 1.6 |

[1]Analysis was stratified by herd.

[2]Hazard ratios were calculated by raising e (2.718) to the $\beta$ power. The hazard ratio estimates the rate of pregnancy for different levels compared with a base level.

[3]0 = 1, 1 = >1.

[4]0 = December to February, 1 = March to May, 2 = June to August, and 3 = September to November.

[5]0 = Rectal palpation (n = 472), 1 = postpartum therapeutic prostaglandin (n = 443), and 2 = scheduled interval prostaglandin (n = 461).

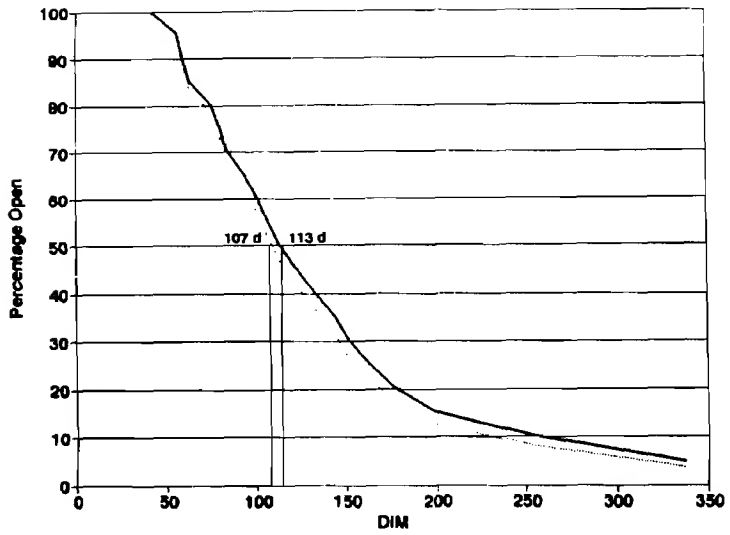


Figure 1. First AI rates (AI per time) in three participating herds for cows on $PGF_{2\alpha}$ at a scheduled interval (---; n = 461) and rectal palpation (—; n = 472) treatments.

the final Cox proportional hazards regression model (Table 4). There was no difference in hazard ratios between PG and RP treatments (Table 5). The hazard ratio for SI treatment was 1.11 when other covariables were controlled. Therefore, after parity group (1 or >1) and season of parturition were controlled, the SI treatment resulted in a hazard ratio of 1.11, indicating that cows under SI treatment had a 11% higher first AI rate than cows under RP treatment. The cows on the SI treatment received three $PGF_{2\alpha}$ injections prior to breed-

TABLE 4. Cox proportional hazard multiple regression model for days to first AI.[1]

| Term | Coefficient[2] | SE | Wald[3] | LRCS[4] |
|---|---|---|---|---|
| Lactation[5] | .01 | .06 | .98 | . . . |
| Season[6] | | | | |
| 1 | −.10 | .09 | .28 | . . . |
| 2 | .13 | .07 | .09 | . . . |
| 3 | .11 | .07 | .14 | . . . |
| Treatment[7] | | | | |
| 1 | −.02 | .07 | .79 | .81 |
| 2 | .12 | .06 | .04 | .04 |

[1]Analysis was stratified by herd.

[2]The coefficients for the categorical variables represent the $\log_e$ of the ratio of hazard functions for different levels compared with the base level.

[3]Wald statistic test is based upon the asymptotic normality of the maximum likelihood estimates.

[4]Likelihood ratio chi-square statistic for treatment.

[5]0 = 1; 1 = >1.

[6]0 = December to February, 1 = March to May, 2 = June to August, and 3 = September to November.

[7]0 = Rectal palpation (n = 472), 1 = postpartum therapeutic prostaglandin (n = 443), and 2 = scheduled interval prostaglandin (n = 461).

ing compared with one $PGF_{2\alpha}$ injection for the cows on the RP treatment. The greater number of $PGF_{2\alpha}$ injections for the SI group resulted in greater synchronization of estrus and earlier DIM to first AI.

Plots of the Kaplan Meier estimators showed median days to first AI for SI treatment to be 63 d compared with 71 d for the RP treatment (Figure 2). Because hazard ratios of PG and RP treatments were not statistically different ($P > .05$), the cumulative first AI rate of PG treatment was not plotted.

#### Descriptive Statistics

Table 6 contains descriptive statistics for reproductive performance that were not important ($P > .10$) among treatments. The effect estimates from the survival analysis differed from the univariable descriptive statistics because they were adjusted for the other variables in the model. By inspection, the cumulative incidence of displaced abomasum, ketosis, milk fever, and retained placenta was not different among treatments (Table 7) and was consistent with results of previous studies (5, 6). By inspection, the cumulative incidence of metritis, ovarian cysts, and pyometra differed among treatments (Table 7), most likely a result of the study protocol, because each of these disorders is diagnosed via rectal palpation, which was prohibited in the PG and SI treatments. In addition, the cumulative inci-

TABLE 5. Hazard ratios and 95% confidence intervals (CI) for days to first AI model.[1]

| Term | Hazard ratio[2] | 95% CI |
|---|---|---|
| Lactation[3] | 1.01 | .7 to 1.2 |
| Season[4] | | |
| 1 | .91 | .6 to 1.5 |
| 2 | 1.14 | .7 to 1.4 |
| 3 | 1.11 | .5 to 1.2 |
| Treatment[5] | | |
| 1 | .98 | .7 to 1.3 |
| 2 | 1.11 | 1.07 to 1.5 |

[1]Analysis was stratified by herd.

[2]Hazard ratios were calculated by raising e (2.718) to the $\beta$ power. The hazard ratio estimates the rate of pregnancy for different levels compared with a base level.

[3]0 = 1; 1 = >1.

[4]0 = December to February, 1 = March to May, 2 = June to August, and 3 = September to November.

[5]0 = Rectal palpation (n = 472): 1 = postpartum therapeutic prostaglandin (n = 443), and 2 = scheduled interval prostaglandin (n = 461).

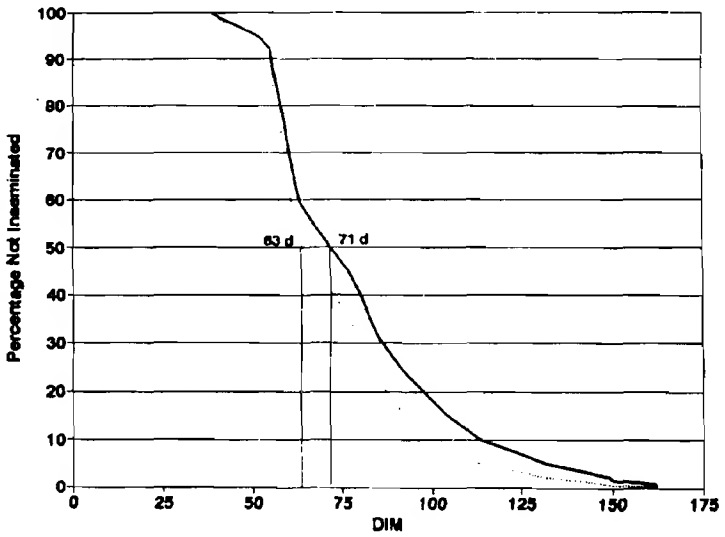


Figure 2. Pregnancy rates (pregnancies per time) in three participating herds for cows receiving $PGF_{2\alpha}$ at a scheduled interval (---; n = 461) and rectal palpation (—; n = 472).

TABLE 6. Univariate descriptive statistics for reproductive performance by treatment.

| Term | RP[1] (n = 472) | | PG[2] (n = 443) | | SI[3] (n = 461) | |
|---|---|---|---|---|---|---|
| | $\bar{X}$ | SD | $\bar{X}$ | SD | $\bar{X}$ | SD |
| First AI conception rate,[4] % | 43 | 2.3 | 45 | 2.4 | 47 | 2.3 |
| Overall conception rate,[5] % | 51 | 2.3 | 53 | 2.4 | 53 | 2.3 |
| Inseminated cows pregnant, % | 88 | 1.5 | 86 | 1.6 | 88 | 1.5 |
| Cows culled,[6] % | 21 | 1.9 | 21 | 2.0 | 20 | 1.8 |
| Days open | | | | | | |
| All cows | 113 | 38 | 114 | 39 | 107 | 29 |
| Pregnant cows | 111 | 43 | 111 | 41 | 104 | 35 |
| AI per conception[7] | 2.0 | 1.0 | 1.9 | 1.0 | 1.9 | .98 |

[1]Rectal palpation treatment.

[2]Postpartum therapeutic $PGF_{2\alpha}$ treatment.

[3]Scheduled interval $PGF_{2\alpha}$ treatment.

[4][(Total conceptions resulting from first AI)/(total cows with a first service)] × 100.

[5][(*AI per conception on all services*)] × 100.

[6][(Total cows culled)/(total cows enrolled)] × 100.

[7]Inseminations per conception on all inseminations.

dence of metritis, ovarian cysts, and pyometra in the RP treatment was consistent with those in previous studies (5, 6). Uterine therapies that were administered were not recorded.

### Partial Budget and Sensitivity Analysis

Costs for PG treatment were $4.46 less per cow than the costs of RP treatment. The SI treatment cost $3.61 less per cow than RP treatment but saved 6 median d open per cow compared with those on RP treatment (Table 8). When the value of a saved day open was assumed to be $2.00/d per cow (9), net costs for the PG treatment did not change, but, for the SI treatment, were reduced from $3.61 to $15.61 less per cow than the costs of RP treatment. Break-even analyses (Table 8) showed that the break-even costs of $PGF_{2\alpha}$ of the SI treatment, with and without postpartum therapeutic costs, were $5.02 and $3.53, respectively. When the price of a dose of

TABLE 7. Cumulative incidence of postpartum disorders by treatment.

| Postpartum disorders[4] | RP[1] (n = 472) | | PG[2] (n = 443) | | SI[3] (n = 461) | |
|---|---|---|---|---|---|---|
| | (%) | (no.) | (%) | (no.) | (%) | (no.) |
| Displaced abomasum | 3.0 | 14 | 2.0 | 9 | 4.0 | 18 |
| Ketosis | 1.3 | 6 | 1.4 | 6 | 1.1 | 5 |
| Metritis[5] | 8.9 | 42 | 0 | 0 | 0 | 0 |
| Milk fever | 2.3 | 11 | 2.0 | 9 | 2.4 | 11 |
| Ovarian cyst[5] | 11.0 | 51 | 3.2 | 14 | 3.3 | 15 |
| Pyometra[5] | .8 | 4 | 0 | 0 | 0 | 0 |
| Retained placenta | 7.6 | 36 | 6.8 | 30 | 7.4 | 34 |

[1]Rectal palpation treatment.

[2]Postpartum therapeutic $PGF_{2\alpha}$ treatment.

[3]Scheduled interval $PGF_{2\alpha}$ treatment.

[4]Clinical cases diagnosed during the postpartum period.

[5]Diagnosis via rectal palpation occurred 25 to 100 d postpartum.

$PGF_{2\alpha}$ varied from \$2.25 to \$3.00 and from \$3.00 to \$4.00, results of the partial budget did not change (Table 8). Combined costs of \$3.00 per dose of $PGF_{2\alpha}$ and a less expensive palpation cost of \$.90 resulted in PG and SI costs of \$1.01 and \$11.87 less per cow, respectively, than costs of RP treatment. Thus, the interpretation of the cost-benefit relationship of using $PGF_{2\alpha}$ as in this study, is dependent on value of days not pregnant, $PGF_{2\alpha}$ costs, and palpation and associated therapy costs.

## DISCUSSION

### Postpartum Therapeutic Tool

A PG program consisting of a therapeutic injection of $PGF_{2\alpha}$ at 25 to 32 d postpartum (RP) was compared with an RP program based on routine RP and intrauterine therapies. A reproductive program consisting of a therapeutic injection of $PGF_{2\alpha}$ at 25 to 32 d postpartum (RP prohibited) may result in equivalent reproductive performance compared with a program based on routine RP and intrauterine therapies. Pregnancy rate, first AI rate, first AI conception rate, overall conception rate, percentage of AI cows that became pregnant, and culling rates were not different between PG and RP treatments. Pregnancy rate is a function of conception rate and efficiency of estrus detection (2, 8, 25, 29). Therefore, reproductive performance was not different among treatments, probably because reproductive performance and efficiency of estrus detection were similar. Although no differences in reproductive performance occurred among treatments for PG and RP, partial budgeting indicated that PG treatment cost was \$4.46 less per cow than the cost of RP treatment.

Results of this comparison are supported by previous research (18, 22, 23, 26, 27) demonstrating that therapies associated with routine RP do not improve reproductive efficiency.

TABLE 8. Partial budget[1] and sensitivity analysis[2] for postpartum therapeutic $PGF_{2\alpha}$ (PG) and $PGF_{2\alpha}$ treatments at scheduled intervals (SI) compared with rectal palpation (RP) treatment.

| Term | RP | PG | SI |
|---|---|---|---|
| Partial budget | | | |
| Number of cows | 472 | 443 | 461 |
| Doses of $PGF_{2\alpha}$[3,4] | 1289 | 1630 | 1890 |
| Cost of $PGF_{2\alpha}$[3,4] at \$2.25 per dose, \$ | 2900 | 3668 | 4253 |
| Rectal palpations,[5] no. | 944 | 0 | 0 |
| Cost of rectal palpation at \$2.25 per palpation, \$ | 2124 | . . . | . . . |
| Cost of postpartum therapy[6] at \$10.88 per treated cow, \$ | 1055 | 435 | 163 |
| Total costs, \$ | 6079 | 4103 | 4416 |
| Cost difference between treatments, \$ | . . . | -1976 | -1663 |
| Cost difference per cow, \$ | . . . | -4.46 | -3.61 |
| Median days open saved per cow, d | . . . | 0 | -6 |
| Value of saved days open at \$2.00/d per cow, \$ | . . . | . . . | -12.00 |
| Net cost per cow (compared with RP treatment), \$ | . . . | -4.46 | -15.61 |
| Sensitivity analysis | | | |
| $PGF_{2\alpha}$[3,4] \$5.02 per dose and days open saved = 0 | . . . | -2.33 | 0 |
| $PGF_{2\alpha}$[3,4] \$3.53 per dose, therapy costs = \$0, and days open saved = 0 | . . . | -2.08 | 0 |
| $PGF_{2\alpha}$[3,4] \$3.00 per dose | . . . | -3.88 | -14.70 |
| $PGF_{2\alpha}$[3,4] \$4.00 per dose | . . . | -3.12 | -13.33 |
| $PGF_{2\alpha}$[3,4] \$3.00 per dose and rectal palpation at \$.90 | . . . | -1.01 | -11.87 |

[1]Using baseline costs.

[2]Net cost per cow compared with costs of RP treatment with the indicated changes.

[3]Includes all postpartum and breeding period injections.

[4]Lutalyse™ (Upjohn, Kalamazoo, MI).

[5]Includes all prebreeding palpations (excludes pregnancy palpations).

[6]Costs (drugs and additional palpations) incurred between 25 to 100 d postpartum.

Therefore, compared with a traditional reproductive program on RP, the use of PG without RP could result in equivalent reproductive performance at lower costs. Because the purpose of our study was to compare the use of PG to traditional RP program in current use, it was outside the scope of our study to test RP alone. Thus, the value of routine RP of all postpartum cows for subsequent reproductive performance was not determined.

#### Tool for Detection of Estrus

The efficacy of administering PG combined with use of $PGF_{2\alpha}$ to synchronize estrus ($PGF_{2\alpha}$ injected at scheduled 14-d intervals after 25 to 32 d postpartum, routine RP prohibited) was compared with a traditional RP program with veterinary intervention (SI vs. RP). Administration of a therapeutic injection of $PGF_{2\alpha}$ combined with use of $PGF_{2\alpha}$ to synchronize estrus is cost-effective and can enhance reproductive performance compared with a traditional RP program. Cows in the SI treatment had higher pregnancy rates and higher rates of first AI, which resulted in fewer days to first AI and fewer days open than for cows receiving a traditional program that relied on routine RP and intrauterine therapies. Improved reproductive performance may be attributed to the greater synchronization of estrus during the postpartum period. Barr (2) established that a decrease of 1 d to first AI corresponds to a decrease of .8 d open. Comparison of SI and RP treatments yielded results (a decrease of 8 median d to first AI, which resulted in a savings of 6 d open in the SI treatment) consistent with those of Barr (2). Rates of first AI conception, overall conception, percentage of AI cows that became pregnant, and culling were not different between cows on SI and RP treatments, which indicates SI and RP cow reproductive performance was similar.

Although the work of Slenning and Farver (31) suggested that administration of $PGF_{2\alpha}$ during breeding lowered the efficiency of estrus detection, SI yielded greater synchronization of estrus prior to breeding, which increased estrous activity during the breeding period (8, 10, 12, 19, 28). Part of the improved reproductive performance of SI treatment was due to the decrease in days to first AI compared with that of cows on the RP treatment. Because conception rates at first AI and overall conception rates were not different among treatments, the fewer days to first AI and to conception and subsequent higher pregnancy rates of SI treatment can be attributed to greater synchronization of estrus, which resulted in earlier AI in SI treatment. In addition, because reproductive performance was not different between PG and RP treatments, the improved reproductive performance of SI treatment compared with RP treatment can be attributed directly to improved synchronization of estrus rather than to postpartum therapeutic effects of $PGF_{2\alpha}$.

The partial budget analysis considered rectal palpation fees, postpartum therapeutic costs, and drug costs for $PGF_{2\alpha}$ across all treatments. Because the potential savings in labor costs for the producer are herd specific, they were not considered. The SI treatment cost $3.61 less per cow than the RP treatment and saved 6 median d open per cow (an additional $12 per cow) compared with RP treatment.

Sensitivity analysis on the partial budget suggested that the decision to use a scheduled interval $PGF_{2\alpha}$ program, such as SI, in a herd would consider economic return of an improved reproductive efficiency (e.g., higher pregnancy rate) in the herd compared with additional drug costs incurred for the program. The benefits of the SI treatment may be less pronounced in herds with either excellent efficiency or poor accuracy of estrus detection and low conception rates.

## CONCLUSIONS

This study evaluated the reproductive performance and net economic benefit of using $PGF_{2\alpha}$ as a postpartum therapeutic and tool for detection of estrus. The PG program was less expensive and resulted in reproductive performance equivalent to that of a RP program based on veterinary intervention. With the SI program, reproductive efficiency and net economic benefit were improved over that of a traditional RP program. The improved reproductive performance associated with an SI program was directly attributed to greater synchronization of estrus, which improved efficiency of detection, rather than to postpartum

therapeutic effects of $PGF_{2\alpha}$. The overall results of this study suggest that a PG program in which $PGF_{2\alpha}$ is used at scheduled intervals is cost effective and may improve herd reproductive performance compared with more traditional programs.

## ACKNOWLEDGMENTS

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# Short Communication: An Electronic Probe Versus Milk Progesterone as Aids for Reproductive Management of Small Dairy Herds

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## ABSTRACT

A simple probe especially designed to take electrical resistance measurements at different positions in the anterior vagina of a cow was compared with milk progesterone determinations on 108 cows. Milk samples were taken 3× weekly, 21 to 60 d postpartum, at the time of insemination, and 21 to 23 d later. Electrical resistance measurements were made on a similar schedule. In 10 other herds, 187 cows had only milk samples taken. No cows with high milk progesterone values became pregnant when inseminated, but the electrical resistance values were less accurate in designating which cows were suitable or unsuitable for insemination. Both low milk progesterone and low electrical resistance values 21 to 23 d after insemination provided an early and accurate indication of a need for reinsemination. These indicators were consistent with 94 to 100% of these cows being diagnosed as not pregnant 6 to 8 wk later. Daily probing, starting about 19 d after a previous insemination, could serve as an early check of pregnancy and assist in identifying cows for immediate reinsemination.
(**Key words**: milk progesterone, vaginal mucus, pregnancy rate)

**Abbreviation key**: **ER** = electrical resistance, **$MP_4$** = milk progesterone.

## INTRODUCTION

Good reproductive management in the postpartum dairy cow continues to be an essential challenge to maintaining a successful dairy enterprise (2, 16). Management aids include monitoring progesterone in the milk by laboratory assays (3, 7, 12, 14) with cowside progesterone kits (1, 13, 15), pedometers (10), and radiotelemetry (17, 18). The rheological properties of vaginal mucus (9) and its electrical conductivity (11) change during the estrous cycle, as does vulvar tissue (8). Milk progesterone (**$MP_4$**) measurements generally are more consistent with other changes in the estrous cycle than are measurements of electrical resistance (**ER**) of vaginal mucus (3, 6). However, when cows that were not visually detected in estrus were inseminated on the basis of ER measurements (5), the pregnancy rate was as high (52%) as for cows inseminated on the basis of a visually detected estrus (49%). Thus, reproductive management of cows, particularly in small herds in northern climates, with limited turnout and without early checks of pregnancy might be improved if inexpensive reliable aids could be provided. No data were available comparing ER and $MP_4$ of postpartum cows from preinsemination through pregnancy in small dairy herds.

The objective of this study was to make a direct comparison of the accuracy and usefulness of taking both dorsal and ventral vaginal ER measurements with an electronic probe specially designed for that purpose versus $MP_4$ assays for monitoring estrous cycles, characterizing cows inseminated, and detecting nonpregnant cows at the next estrus. Nonpregnant cows could be reinseminated at the next estrus in small dairy herds.

## MATERIALS AND METHODS

### Cows

Twenty Holstein cows at Cornell University and 39 cows in two nearby herds (A, B) on DHI, with only AI and with regular veterinary care, were sampled with both a vaginal ER probe and $MP_4$ assays. However, only 33 cows in herds A and B had sufficient comparable information to be included in the final analysis. Also, milk samples were taken, but no probing was done in 187 cows in 10 other nearby herds (herds C to L) on DHI that were visited biweekly by the Ambulatory Clinic from the New York State College of Veterinary Medicine (Ithaca, NY). Both $MP_4$ and ER were assessed for 55 cows in one additional herd (M) on

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DHI and with veterinary service in northern New York. These herds were managed according to their standard practices, and reproductive records obtained later were compared with the experimental data. Cows were checked for pregnancy by palpation of the reproductive organs per rectum 6 to 8 wk after insemination.

### Milk Sampling Procedure

Milk samples after milking machine removal (last milk samples) were collected at Cornell University and from herds A, B, and M on Monday, Wednesday, and Friday from 21 to 60 d postpartum. Samples were collected from herds C to L 35 to 60 d postpartum, as well as on the day of insemination and 21 and 22 or 23 d after insemination. Progesterone is higher in last milk samples than in composite milk samples (4), and if a last milk sample is missed during milking, it can be taken a short time later.

### Electronic Probe

The probe had two pairs of axillary parallel stainless steel electrodes, 90 degrees apart, embedded in the tip. A switch on the handle of the probe allowed the operator to activate the dorsal or ventral pair of electrodes separately. Thus, after positioning the tip of the probe about 2 cm from the cervix, ER across the ventral electrodes was taken. The probe was held so that the tip was believed to be approximately central in the anterior vagina with the ventral electrodes in any mucus that might be pooled just posterior to the cervix. The ER across the dorsal electrodes was taken with the tip of the probe in a similar position against the floor of the vagina with the dorsal electrodes in the mucus but away from the ventral vaginal mucosa. Care was taken to sanitize the probe after each set of measurements, and use of the moist sanitized probe caused no vaginitis. There were 2009 ER measurements taken with ventral electrodes, and 2009 were taken with dorsal electrodes.

### Progesterone Determinations

About 4900 milk samples were collected from the 108 cows with ER measurements and from the 187 cows without ER measurements. Samples were placed in a freezer at -20°C until assayed. Milk samples were extracted with petroleum ether. The aqueous portion was frozen in a methanol-dry ice bath and discarded. The organic fraction was dried and redissolved in 70 to 80% methanol and was extracted twice with petroleum ether (4) and dried. Progesterone was determined by a standard radioimmunoassay procedure (6).

### Analysis of Data

Estrous cycle information, calving dates, dates of insemination, and results of checks of pregnancy by rectal palpation 6 to 8 wk after the last insemination were compared with ER and $MP_4$ data. To determine whether the experimental ER and $MP_4$ results would be useful for making decisions regarding insemination and pregnancy status, the experimental values were divided in advance into low and high groups, based on preliminary tests and previous experiments (4, 5, 6). The low $MP_4$ values were $\leq$0.3 ng/10 $\mu$l of extracted milk, and high $MP_4$ values were >0.3 ng/10 $\mu$l. Low ventral and dorsal electrode ER values were set at $\leq$30 ohms, and high values were set at >30 ohms. The cows in the low value groups at the time of insemination were expected to have a higher probability of becoming pregnant than were those in the high value groups. Low values at 21 to 23 d postinsemination were expected to indicate that a cow was not pregnant. The ratios of cows in the expected and observed groups were compared statistically by chi square.

## RESULTS

### $MP_4$ and ER

Differences among herds for $MP_4$ and ER measurements were generally not significant, and the results for 53 cows in the Cornell herd and herds A and B, probed by the same person, are combined in Table 1. Also, surprisingly, the dorsal and ventral ER values were nearly identical, and so they were averaged for analysis. Those cows with low $MP_4$ values at the time of insemination were more likely to become pregnant than were those with high values ($P < 0.05$), but the ER measurements did not relate well to the eventual pregnancy status. At 21 to 23 d postinsemination all cows with low $MP_4$ or ER values were later found to be not pregnant, as predicted. Some of the cows with high $MP_4$ and ER values were pregnant, but also many cows with high values were later found to be nonpregnant.

In herd M, the ER values for individual cows taken by a person at the farm varied and tended to be lower on the day before insemination. The mean ER values 2 and 1 d before insemination and on the day of insemination for cows that became pregnant (43, 31, and 38 ohms, respectively) were not different ($P > 0.05$) from cows that did not conceive (43, 30, and 38

TABLE 1. Grouping of cows from three herds by pregnancy status based on milk progesterone ($MP_4$) and electrical resistance (ER) values at the time of insemination and 21 to 23 d later.

| Time of sampling and pregnancy status later | Number of cows with high or low values | | | |
|---|---|---|---|---|
| | $MP_4$ level | | ER values | |
| | Low | High | Low | High |
| At insemination | | | | |
| Pregnant | 18 | 0[a] | 11 | 7[a] |
| Not pregnant | 21 | 6[b] | 15 | 12[a] |
| 21 to 23 d later | | | | |
| Pregnant | 0 | 20[a] | 0 | 20[a] |
| Not pregnant | 20 | 13[b] | 10 | 23[b] |

[a,b]Paired ratios are different; $P < 0.05$.

ohms, respectively). However, 91% of the ER values for 55 cows (herd M) were low when $MP_4$ values were low. When $MP_4$ was low, 81% of the cows were reported in estrus, and 57% conceived at insemination. On d 21 to 23, 100% of the cows with low $MP_4$ or low ER values were later diagnosed as not pregnant.

In herds C to L the overall pregnancy rate for 187 cows was 55%. For cows checked 21 to 23 d after insemination, the low $MP_4$ determinations were in agreement with the nonpregnant condition of cows in 98% of the cases. For cows checked later and found to be pregnant, $MP_4$ values on d 21 to 23 were in 80% agreement.

## DISCUSSION

The volume of cervical mucus in the anterior vagina increases, and ER of the anterior vagina and vulvar tissue decreases at the time of estrus in cattle (8, 9). If the farm is equipped with an appropriate probe, the ER can be measured on successive days quickly and cheaply. The probe and sanitizing unit used here were similar to units described previously (5) and cost about $200 to construct. Twenty cows in loafing areas or stanchions can be probed per hour, and the probe can be sanitized between cows with a simple sanitizer (5). Cows inseminated when the ER values are lowest are expected to be near the appropriate stage of the estrous cycle for insemination (6). Thus, these values may assist in detecting cows suitable for insemination under conditions where detection of behavioral estrus is difficult or signs are inconclusive. Likewise, low $MP_4$ values should indicate that the cow is near the estrous phase, but low $MP_4$ values occur for a longer time than do ER values, so multiple measurements are necessary.

The $MP_4$ measurements for the 301 cows studied in all herds demonstrated that no cows with high $MP_4$ values at insemination became pregnant. The ER values were more variable and less consistent with eventual pregnancy status. Part of this inconsistency could have been due to the short time that ER values were low. As values were often rising by the time of insemination, pregnancy could have occurred with ER values classified as high. This phenomenon could account for the differing values compared with the previous results obtained when cows were inseminated at a low ER reading (6).

High $MP_4$ and ER values 21 to 23 d after insemination frequently were associated with cows diagnosed as not pregnant 6 to 8 wk after insemination. A portion of this discrepancy likely is due to early embryonic death in some cows (7). Also, cows inseminated at the wrong stage of the estrous cycle would be in diestrus 3 wk later.

The low $MP_4$ and ER values 21 to 23 d after insemination provided the most consistent information for management decisions, as 94 to 100% of the values in the different groups of herds were in agreement with cows being found not pregnant. These percentages are similar to those reported by Nebel et al. (13) who used less quantitative cowside tests.

Changes in ER of vaginal mucus (3, 5, 6) or in vulvar tissue (8) occur more abruptly than do the changes in concentrations of $MP_4$. Therefore, measurement of ER has an advantage over determination of $MP_4$ in that the number of samples required is lower for estimation of when to inseminate. For example, all cows, or cows with long intervals from calving to first service, could be probed daily starting 19 d after the previous service. This check could serve both as a check of pregnancy and an indication of when to reinseminate without delay, particularly when fresh mucus is observed on the tip of the probe (5, 6).

Such a program would have very little operational cost, except for some labor, and could be an inexpensive adjunct for improving reproductive management where weather and housing limit visual detection of estrus and where early checking for pregnancy is not done. The costs versus economic benefits of such a system seem worthy of simulation as has been done with cowside $MP_4$ tests (16).

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# Scanning the Future—Ultrasonography as a Reproductive Management Tool for Dairy Cattle[1]

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## ABSTRACT

Application of transrectal real-time ultrasonography as a research tool to study bovine reproduction represents a technological breakthrough that has revolutionized our understanding of reproductive biology in cattle. The widespread adoption and use of ultrasonography for routine reproductive examinations of dairy cattle by bovine practitioners is the next contribution this technology will make to the dairy industry. Assessment of pregnancy status and fetal viability early postbreeding to identify cows that fail to conceive improves reproductive efficiency by decreasing the interval between artificial insemination services and increasing artificial insemination service rate. Early identification of cows carrying twin fetuses will allow for implementation of differential management strategies to abrogate negative effects of twinning during the periparturient period once such strategies have been developed. Ovarian and uterine pathologies not accurately detected via rectal palpation can easily be visualized by ultrasound, and appropriate therapies can be implemented. Determination of fetal sex in utero is useful when coupled with a management decision that justifies the expense of fetal sexing. Development of integrated reproductive management systems that combine ultrasound with new and existing reproductive technologies will further enhance the practical applications of ultrasonography. Development of Extension education programs to train bovine practitioners to use ultrasound for routine reproductive examinations is a critical step toward rapid implementation of this technology into the dairy industry.

(**Key words:** ultrasound, dairy cattle, reproductive management)

## INTRODUCTION

The application of transrectal real-time ultrasonography to the study of bovine reproduction represents a technological breakthrough that has revolutionized knowledge of reproductive biology. New research information generated through ultrasonic imaging has clarified the nature of complex reproductive processes in cattle including ovarian follicular dynamics, corpus luteum function, and fetal development. Early integration of ultrasound technology to the dairy industry included applications such as transvaginal follicular aspiration and oocyte recovery (Pieterse et al., 1988; Pieterse et al., 1991; Meintjes et al., 1993) and as a complementary technology for embryo transfer procedures. These applications are, however, specialized and will not likely constitute widespread use of ultrasound technology in the dairy industry. The purpose of this review is to examine practical applications of ultrasound to the dairy industry that may constitute widespread use of this technology in the future.

Practical applications of ultrasound include early assessment of pregnancy status, identification of cows carrying twin fetuses, detection of ovarian and uterine pathologies, and determination of fetal sex. Each of these applications presents opportunities for improving reproductive efficiency on a dairy farm. Unfortunately, most veterinary students continue to be taught that ultrasound is a secondary technology for bovine reproductive work; however, the information-gathering capabilities of ultrasonic imaging far exceed those of rectal palpation (Ginther, 1995). Extension education programs to train bovine practitioners to use ultrasound for routine reproductive examinations are a critical step toward rapid implementation of this technology into the dairy industry.

### Veterinary Ultrasound Equipment

Detailed information on the principles of ultrasonography is beyond the scope of this review and has been reviewed elsewhere (Ginther, 1995). In general, linear-array, real-time, B-mode (brightness modality) ultrasound scanners are best suited for veterinary applica-

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tions involving dairy cattle reproduction. Most ultrasound machines consist of a console unit that contains the electronics, controls, and a screen upon which the ultrasound image is visualized by the operator, and a transducer, which emits and receives high-frequency ultrasound waves. Linear-array transducers consist of a series of piezo electric crystals arranged in a row. These crystals emit high frequency sound waves upon being energized. The configuration of a linear-array transducer results in a rectangular image on the field of scan (as opposed to a pie shaped image produced by a sector transducer). Currently, a veterinary-grade ultrasound machine equipped with one rectal transducer can be purchased for $8000 to $16,000. Image quality varies widely among veterinary-grade ultrasound machines and generally increases with the cost of the unit. More expensive machines are available but are not necessary for routine reproductive examinations in dairy cattle.

Bovine reproductive organs are most commonly scanned per rectum using a linear-array transducer specifically manufactured for transrectal use. However, specialized applications including ovum pickup and follicle ablation involve a transvaginal approach using a sector transducer. Linear-array transducers of 5.0 and 7.5 MHz frequency ranges are most commonly used in cattle to perform reproductive ultrasound examinations, and most veterinary ultrasound scanners are compatible with probes of different frequencies. The depth of tissue penetration of sound waves and image resolution is dependent upon and inversely related to the frequency of the transducer. Thus, a 5.0-MHz transducer results in greater depth of tissue penetration and lesser image detail, whereas a 7.5-MHz transducer results in lesser depth of tissue penetration and greater image detail. An ultrasound scanner equipped with a 5.0-MHz transducer is most useful for bovine practitioners conducting routine reproductive examinations; however, small ovarian structures such as developing follicles are best imaged with a 7.5-MHz transducer.

## IMAGING THE BOVINE OVARY

### Ovarian Follicles

Folliculogenesis is the process of forming mature follicles capable of ovulation from the pool of nongrowing, primordial follicles in the ovary (Spicer and Echternkamp, 1986). Ovarian follicles are fluid-filled structures surrounded by an inner layer of granulosa cells and an outer layer of thecal cells. The oocyte is suspended within the antrum by a specialized pedicle of granulosa cells called the cumulus oophorous. Because fluid absorbs rather than reflects ultrasound waves, fluid-filled structures such as antral follicles appear as black circular structures surrounded by echogenic ovarian tissue. Most veterinary grade ultrasound scanners can resolve ovarian follicles with a diameter of 2 to 3 mm or greater, and larger antral follicles can easily be tracked during serial scanning sessions (Pierson and Ginther, 1988).

### Corpora Lutea

The CL is a transient endocrine gland that forms after ovulation from the tissues that previously constituted the ovarian follicle. Thus, the CL can be viewed as the terminal stage of follicular development. Corpora lutea appear as distinctly echogenic areas within the ovarian stroma. Many corpora lutea appear as a solid tissue masses but may also contain fluid-filled cavities. Based on ultrasonographic examinations in dairy heifers, 79% of otherwise normal CL contain cavities ranging from less than 2 to greater than 10 mm in diameter at some time during the estrus cycle and early pregnancy (Kastelic et al., 1990b; Singh et al., 1997). Ovarian cysts containing luteinized tissue should not be confused with a normal CL containing a fluid-filled cavity.

Ultrasonographic attributes of CL including cross-sectional diameter, luteal area, and echogenicity have been correlated to luteal structure and function (Kastelic et al., 1990a; Singh et al., 1997; Battocchio et al., 1999). Use of luteal characteristics to improve the accuracy of pregnancy diagnosis has been reported in dairy heifers (Kastelic et al., 1991), but similar data have not been reported for lactating dairy cows. Luteal size and echogenic characteristics assessed at specific times postbreeding may prove useful as a method to improve the accuracy of early pregnancy diagnosis in dairy and beef cattle. Although ultrasound is more accurate than rectal palpation for assessing ovarian follicles, it is difficult to distinguish between developing corpora lutea and older regressing corpora lutea using either technique (Pieterse et al., 1990b).

### Ovarian Cysts

Currently, diagnosis of cysts in cattle most often occurs during routine postpartum rectal examinations conducted by a bovine practitioner. Palpation per rectum of a large, fluid-filled structure is commonly used as a clinical indication of a follicular cyst. Differentiation between follicular and luteal cysts via rectal palpation is difficult, even for experienced practitioners (Dawson, 1975; Farin et al., 1992). Accuracy of diagnosis increases when using transrectal ultrasonography, with correct identification of greater than 90% of luteal and nearly 75% of follicular cysts (Farin et al., 1990, 1992).

**Table 1.** Day of first detection of ultrasonographically identifiable characteristics of the bovine conceptus.[1]

| Characteristic | First day detected | |
|---|---|---|
| | Mean | Range |
| Embryo proper | 20.3 | 19 to 24 |
| Heartbeat | 20.9 | 19 to 24 |
| Allantois | 23.2 | 22 to 25 |
| Spinal cord | 29.1 | 26 to 33 |
| Forelimb buds | 29.1 | 28 to 31 |
| Amnion | 29.5 | 28 to 33 |
| Eye orbit | 30.2 | 29 to 33 |
| Hindlimb buds | 31.2 | 30 to 33 |
| Placentomes | 35.2 | 33 to 38 |
| Split hooves | 44.6 | 42 to 49 |
| Fetal movement | 44.8 | 42 to 50 |
| Ribs | 52.8 | 51 to 55 |

[1]Adapted from Curran et al., 1986.

### Ovarian Structures as Diagnostic Aids

A thorough reproductive examination in cattle includes visualization of the major structures (or the lack thereof) on both ovaries. Although rectal palpation can be an accurate method for diagnosing pregnancy, rectal palpation is a poor method for resolving ovarian follicles (Pieterse et al., 1990b). By contrast, ultrasonic imaging is a highly accurate and rapid method for assessing ovarian structures (Griffin and Ginther, 1992). Too often, bovine practitioners proceed directly to scanning the uterus during reproductive examinations and neglect the ovaries all together. This is unfortunate because the ovaries contain a wealth of information that can be used to aid in diagnosing the reproductive status of the cow and for selecting appropriate therapies or reproductive interventions. For example, the presence or absence of a corpus luteum aids in diagnosing pregnancy status, especially when conducting pregnancy exams early postbreeding. When present, the size and location (i.e., left vs. right ovary) of the corpus luteum indicate the location of the conceptus within the uterus if the cow is pregnant. Because most twinning in cattle is dizygotic (Fricke, 2001), the presence of multiple corpora lutea is a diagnostic indicator for cows at risk for conceiving twins. Ovarian pathologies and dysfunction including "static ovaries" (e.g., ovaries of anovular cows with no discernable follicular or luteal structures) and follicular and luteinized cysts can easily be distinguished.

## IMAGING THE BOVINE UTERUS AND CONCEPTUS

Detection of the embryo proper as well as embryonic and fetal developmental characteristics during early fetal development are shown in Table 1. The bovine fetus can be visualized beginning at 20 d postbreeding and continuing throughout gestation. However, because of its size in relation to the image field of view, the fetus cannot be imaged in toto after about 90 d with a 5.0-MHz linear-array transducer.

### Early Pregnancy Diagnosis

The use of transrectal ultrasonography to assess pregnancy status early during gestation is among the most practical applications of ultrasound for dairy cattle reproduction. Early identification of nonpregnant cows postbreeding improves reproductive efficiency and pregnancy rate in cattle by decreasing the interval between AI services and increasing AI service rate. Pregnancy diagnosis in dairy heifers based on the presence of intraluminal uterine fluid before d 16 is unreliable because small amounts of fluid are present in noninseminated heifers as early as d 10; however, accuracy of diagnosis based on fluid alone approached 100% by d 20. The accuracy of pregnancy diagnosis in dairy heifers was not greater than 50% before d 18 with a 5.0-MHz transducer, or before d 16 with a 7.5-MHz transducer (Kastelic et al., 1991).

Under most on-farm conditions, pregnancy diagnosis can be rapidly and accurately diagnosed using ultrasound as early as 26 d post AI (Kastelic et al., 1991; Filteau and DesCôteaux, 1998). Sensitivity and specificity of pregnancy diagnosis with ultrasound was 44.8 and 82.3%, respectively, when conducted between 21 and 25 d post AI but increased to 97.7 and 87.7%, respectively, when conducted between 26 and 33 d post AI (Pieterse et al., 1990a). Although pregnancy status can be established early, care must be taken to ensure the accuracy of a diagnosis. For example, a false-negative diagnosis was more likely when the uterus was located cranial to the pelvic inlet in beef cattle than when the uterus was within the pelvic cavity (Szenci et al., 1995).

Ultrasound is a rapid method for pregnancy diagnosis, and experienced palpators adapt to ultrasound technology quickly. The time required to assess pregnancy in beef heifers at the end of a 108-d breeding season averaged 11.3 s using palpation per rectum versus 16.1 s required to assess pregnancy and fetal age using ultrasound (Galland et al., 1994). Fetal age also affected time required for diagnosis with older fetuses requiring less total time for diagnosis (Galland et al., 1994). Although ultrasound at ≥45 d of gestation did not increase the accuracy of pregnancy diagnosis for an experienced palpator, it may improve diagnostic accuracy of a less experienced one (Galland et al., 1994). Generally, two factors affect the speed at which ultrasound examinations can be conducted on a dairy farm: operator proficiency and availability and restraint of animals. When

both factors are optimized, the speed of ultrasonography can approach that of palpation, while exceeding palpation in the amount of information gathered from each animal.

## Early Embryonic Loss

Pregnancy loss contributes to reproductive inefficiency because fertility assessed at any point during pregnancy is a function of both conception rate and pregnancy loss. Conception rates at 28 to 32 d post AI in lactating dairy cows range from 40 to 47% (Pursley et al, 1997; Fricke et al., 1998), whereas, conception rates in dairy heifers are nearly 75% (Pursley et al., 1997). Similarly, pregnancy loss in lactating dairy cows is greater than that in dairy heifers (20 vs. 5%; Smith and Stevenson, 1995). Although the specific factors responsible for early embryonic loss in dairy cows are not known, they may be similar to those factors responsible for reduced conception rates.

Because pregnancy status can be determined earlier by ultrasound compared with palpation, the rate of pregnancy loss detected is often higher when a positive diagnosis is made earlier postbreeding. Of cows diagnosed pregnant at 28 d post AI, 10 to 16% experience early embryonic loss by 56 d post AI (Mee et al., 1994; Vasconcelos et al., 1997; Fricke et al., 1998). Therefore, cows diagnosed pregnant at 28 d post AI using ultrasound should be scheduled for a subsequent examination around 60 d post AI, when the rate of embryonic loss per day decreases dramatically (Vasconcelos et al., 1997). Although the rate of pregnancy loss is significant in studies using ultrasound to assess the rate of loss, the technique of ultrasound itself has not been implicated as a cause of embryonic death in cattle (Ball and Logue, 1994; Baxter and Ward, 1997). Furthermore, ultrasound is a much less invasive technique for early pregnancy diagnosis than is rectal palpation (Paisley et al., 1978; Vaillancourt et al., 1979) and may minimize the rare incidence of palpation-induced abortions.

At present, there is no practical way to reduce early embryonic loss in lactating dairy cows. However, recognizing the occurrence and magnitude of early embryonic loss may actually present management opportunities by taking advantage of new reproductive technologies that increase AI service rate in a dairy herd. If used routinely, transrectal ultrasonography has the potential to improve reproductive efficiency within a herd by reducing the period from AI to pregnancy diagnosis to 26 to 28 d with a high degree of diagnostic accuracy (Pieterse et al., 1990a). Early pregnancy diagnosis can, however, only improve reproductive efficiency when a nonpregnancy diagnosis is coupled with a management strategy to rapidly return cows to AI service, and such strategies have not yet been empirically tested for their effects on conception rate. In addition, cows diagnosed pregnant at an early ultrasound exam have a greater risk of early embryonic loss and, therefore, must undergo subsequent pregnancy examinations to identify and rebreed cows that experience such loss. If left unidentified, cows experiencing embryonic loss after an early pregnancy diagnosis would actually reduce reproductive efficiency by extending their calving interval. This concept applies not only to ultrasound, but also to any method used to assess pregnancy status early post breeding.

## Identification of Cows Carrying Twins

Twinning is an unavoidable outcome of reproduction in dairy cattle and is undesirable because it reduces overall dairy farm profitability and reproductive efficiency (Eddy et al., 1991; Beerepoot et al., 1992). The reported incidence of twinning in dairy cattle ranges from 2.5 to 5.8% and is dramatically affected by parity, ranging from 1% for first parity to nearly 10% during later parities (Table 2).

Cows carrying twin pregnancies can be accurately identified using transrectal ultrasonography by 40 to 55 d post AI (Echternkamp and Gregory, 1991; Davis and Haibel, 1993; Dobson et al., 1993). When conducting an early diagnosis for twins, the entire length of both uterine horns must be carefully scanned to ensure that an embryo is not missed. Because the majority of twinning in dairy cattle occurs due to double ovulations (Wiltbank et al., 2000), the presence of two or more CL on the ovaries at the time of pregnancy diagnosis is an excellent indicator of cows with an increased risk of conceiving twins. Overall, the incidence of double ovulation in lactating dairy cows after synchronization of ovulation using Ovsynch was 14.1% (Fricke et al., 1998), and the frequency of double ovulation was nearly threefold greater for cows with greater than average milk production near the time of AI than for cows with less than average milk production near the time of AI (Fricke and Wiltbank, 1999).

Several management scenarios could be considered upon identification of a cow carrying twins including culling, abortion and rebreeding, or continued management until parturition (Fricke, 2001). Continued management of the cow could be avoided either by culling the cow or by aborting the twin pregnancy, usually through administration of an ecbolic agent such as $PGF_{2\alpha}$. Several factors would argue against aborting a twin pregnancy with the intent of rebreeding the cow. First, although the calving interval would vary widely among cows subjected to abortion, the estimated average calving interval of cows subjected to induced abor-

**Table 2.** Effect of parity on twinning rate (%) in dairy cattle.[1]

| Ref[2] | No. of calvings | Parity | | | | | | All parities |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| 1 | 937 | 0.7 | 5.0 | 4.2 | 5.0 | 7.0 | 6.7[3] | 4.2 |
| 2 | 7387 | 1.3 | 4.4 | 5.3 | 4.6 | 5.8 | 6.0 | 4.6 |
| 3 | 11,951 | 0.8 | 2.7 | 4.1 | 4.5 | 4.9 | 4.8[3] | 3.2 |
| 4 | 19,755 | 0.9 | 2.1 | 3.5 | 3.4 | 3.7 | 3.2 | 2.5 |
| 5 | 24,843 | 1.0 | 7.0 | 7.5 | 7.9 | 9.1[b] | . . . | 4.2 |
| 6 | 19,497 | 1.3 | 6.0 | 9.4[b] | . . . | . . . | . . . | . . . |
| 7 | 52,362 | 1.0 | 2.9 | 3.2 | 3.9 | 3.3 | 4.1[b] | 2.4 |

[1]Adapted from Wiltbank et al., 2000.

[2]Reference: 1 = Pfau et al., 1948; 2 = Erb and Morrison, 1959; 3 = Nielen et al., 1989; 4 = Eddy et al., 1991; 5 = Ryan and Boland, 1991; 6 = Berry et al., 1994; 7 = Kinsel et al., 1998.

[3]Includes all cows ≥ the parity listed.

tion and rebreeding would exceed 500 d (~18 mo) based on average reproductive performance and management indices for lactating cows (Fricke, 2001). Second, the risk for a twin pregnancy during the subsequent gestation is increased because cows calving twins are at greater risk for subsequent twinning (Nielen et al., 1989). Third, establishing pregnancy in lactating dairy cows is difficult, and a pregnancy represents an inherent value to the dairy farm that is forfeited by electively aborting the pregnancy. Finally, cows carrying twins experience greater rates of early embryonic loss than cows carrying singletons and, on occasion, loose one fetus while maintaining the other (Day et al., 1995). Elective abortion of a twin pregnancy early during gestation that may result in the birth of a singleton calf at parturition is not a sound management practice. Based on these considerations and depending on the value of the dam and calf, culling to avoid continued management of a cow carrying twins is a better alternative to aborting the pregnancy. At present, information on twinning management in dairy cattle is inadequate, and further research is needed to make sound management decisions regarding twinning (Fricke, 2001).

### Determination of Fetal Sex

Transrectal ultrasound can be used to detect the sex of bovine fetuses in utero. Sex is determined by evaluating the morphology and location of the genital tubercle using ultrasound and is a reliable and accurate method for sex determination beginning on d 55 to 60 of gestation (Curran et al., 1989). Because the reproductive tract and conceptus descend beyond the pelvic rim and into the abdominal cavity as gestation ensues, it becomes increasingly difficult to physically reach the fetus using a transrectal approach during later stages of gestation. Generally, a greater level of operator experience and proficiency is required for sex determination using ultrasound compared with that required for early pregnancy diagnosis or examination of ovarian structures.

In a production dairy system, determination of fetal sex is useful when combined with a management decision or strategy that justifies the expense of fetal sexing. In other words, a dairy producer who pays for information regarding fetal sex must economically justify the usefulness of that information. Fulfilling sales contract obligations regarding the sex of a calf carried by a pregnant cow to be sold is one scenario that may justify this expense. If the sex of a calf is a determining factor for culling decisions regarding a pregnant cow, fetal sexing might also be justified. In contrast, the cost associated with fetal sexing is unwarranted if the information is not used to make a management decision. Because of the economic and management considerations associated with fetal sexing, routine fetal sexing of all pregnant cows in a herd will not likely become a standard reproductive management practice unless bovine practitioners choose to conduct fetal sexing at little or no additional expense beyond pregnancy diagnosis.

## DIAGNOSTIC LIMITATIONS OF ULTRASONIC IMAGING

Under most circumstances, practical application of ultrasound for routine reproductive management on a dairy farm consists of a single ultrasound examination conducted at a given point in time as opposed to serial ultrasound examinations. The physiological status of a follicle (e.g., dominant, subordinate, growing, regressing) or corpus luteum cannot be determined during a single ultrasound examination. In addition, ultrasonic imaging aids in distinguishing anatomical attributes of a structure but confers little information regarding physiological or endocrine status. For example, ovarian cysts can be categorized by anatomical attributes such as diameter and presence or absence of

luteal tissue; however, information regarding functionality such as plasma hormone concentrations cannot be conferred. One exception would be the visualization of a fetal heartbeat as a diagnostic indicator of a viable fetus. The diagnostic limitation of ultrasonic imaging becomes important especially when the limitation is exceeded and an incorrect therapy or reproductive intervention is recommended. A thorough understanding of ovarian physiology and the mechanisms by which hormonal programs succeed or fail is critical for correct interpretation of ultrasonic imaging information.

## ECONOMIC CONSIDERATIONS OF ULTRASOUND

Few economic analyses on the use of ultrasonography for reproductive management of dairy cattle have been published to date. However, economic assessment of strategies for early detection of nonpregnancy using other technologies including milk progesterone tests, pressure-mounted heat detection devices, and rectal palpation showed that the earlier nonprenancy was accurately and efficiently detected, the greater the economic return (Oltenacu et al., 1990). Because nonpregnancy can be established 7 to 14 d earlier post AI when using ultrasound compared with rectal palpation, nonpregnant cows can be detected earlier and returned to AI service, thereby improving the pregnancy rate through an increased AI service rate. Thus, early identification of nonpregnancy post AI coupled with rapid return of nonpregnant cows to AI service may be an economically profitable strategy for a dairy farm.

Ultrasound has previously been reported to be an economically profitable reproductive management strategy for dairy farms (DesCôteaux, and Fetrow, 1998). Economic models were structured to evaluate the annual impact of ultrasonography in reducing average days open. The models included the cost and depreciation of ultrasound equipment, 70% pregnant cows at initial pregnancy examinations, and a cost of each day open past 100 DIM of $4.00. The estimated breakeven costs of ultrasound examinations conducted weekly, bimonthly, and monthly were $8.40, $16.80, and $36.00, respectively (DesCôteaux, and Fetrow, 1998). These authors concluded that experienced veterinarians could easily pay back their investment in an ultrasound machine within 3 yr when charging half of the breakeven cost of ultrasound while servicing 15 well-managed 100-cow dairies (DesCôteaux, and Fetrow, 1998). Furthermore, as the proportion of pregnant cows at pregnancy evaluation decreases below 70%, the economic impact of ultrasound increased (DesCôteaux, and Fetrow, 1998). Despite this report, however, a more thorough evaluation of the economics of ultrasound use in conjunction with timed insemination protocols must be undertaken before use of ultrasound can be justified for reproductive management of dairy cattle.

## INTEGRATION OF ULTRASOUND INTO THE DAIRY INDUSTRY

### Integrated Reproductive Management Strategies

New technologies to identify nonpregnant dairy cows early post AI may play a key role in a reproductive management strategy for commercial dairy farms. When using ultrasound for early pregnancy diagnosis, emphasis must be given to identifying nonpregnant rather than pregnant cows. Coupling a nonpregnancy diagnosis with a management decision to quickly reinitiate AI service improves reproductive efficiency and pregnancy rate by decreasing the interval between AI services, thereby increasing AI service rate. Because AI conception rates of high producing lactating dairy cows are reported to be 40% or less (Pursley et al., 1997; Fricke et al., 1998), 60% or more of cows will fail to conceive to an AI service and, therefore, will require a resynchronization strategy for aggressively initiating a subsequent AI service. Ovsynch, a protocol for synchronizing ovulation in lactating dairy cows, uses injections of GnRH and $PGF_{2\alpha}$ (Pursley et al., 1995, 1997) and is an effective method for hormonally programming cows to receive a timed AI service. Hormonal resynchronization systems that program nonpregnant cows to receive a subsequent AI service need to be developed and assessed to aggressively manage reproduction in lactating dairy cows.

The upper protocol in Figure 1 shows a scenario for combining use of Ovsynch and early pregnancy diagnosis using ultrasound. Groups of cows past the voluntary waiting period would receive their first postpartum insemination after synchronization of ovulation using Ovsynch. This would dramatically reduce median days to first AI by eliminating estrus detection for the first postpartum breeding. On d 25 post AI, nonpregnant cows would be identified using ultrasound, and would receive the first GnRH injection for resynchronization using Ovsynch. This would result in an average interval between services of 35 d for cows requiring resynchronization. The lower protocol in Figure 1 shows a more aggressive scenario for combining use of Ovsynch and early pregnancy diagnosis using ultrasound. Groups of cows past the voluntary waiting period would receive their first postpartum insemination after synchronization of ovulation using Ovsynch. On d 18 post AI, all cows would receive an injection of GnRH regardless of their pregnancy status. Ultrasound would be used on d 25 to identify nonpregnant cows, which would receive a $PGF_{2\alpha}$ injection for resynchronization using Ovsynch.

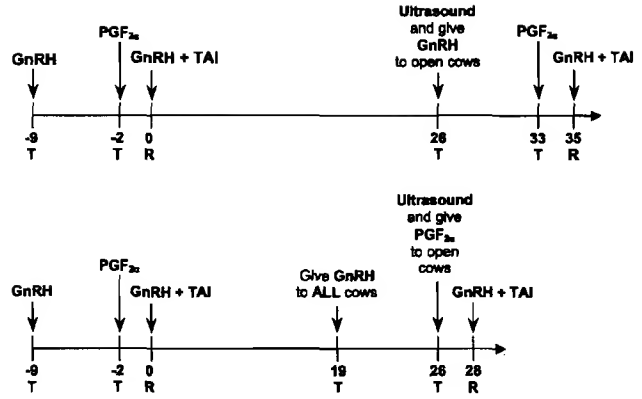


**Figure 1.** Two theoretical strategies for aggressive reproductive management protocols for combining timed AI (Ovsynch) with early pregnancy diagnosis using ultrasound. Note that hormone injections are scheduled for Tuesdays (T) and Thursdays (R), ultrasound examinations for Tuesdays (T), and timed AI (TAI) for Thursdays (R). Average interval between timed AI services would be 35 d (upper protocol) or 28 d (lower protocol) for cows requiring resynchronization. Note that these protocols are meant to illustrate the potential for aggressive resynchronization of nonpregnant cows after an initial timed AI service and must first be empirically tested before they are implemented on dairy farms.

It is important to note that administration of GnRH to pregnant cows for resynchronization of ovulation after a timed AI has been shown to increase early embryonic loss compared with pregnant cows not receiving GnRH (Moreira et al., 2000b). Conception rate and embryonic loss may vary due to the stage of follicular development at the time of resynchronization using GnRH similar to that shown for initiation of Ovsynch during the estrus cycle in dairy cattle (Moreira et al., 2000a; Vasconcelos et al., 1999). Thus, the protocols shown in Figure 1 must first be empirically tested before they are implemented in the dairy industry and are meant to illustrate the potential for aggressive resynchronization of nonpregnant cows after an initial timed AI service. Further research into the efficacy of protocols that combine timed AI with ultrasonography for aggressive reproductive management of dairy cattle is under way.

## The Role of Bovine Practitioners

Currently, the use of ultrasound by bovine practitioners for routine reproductive management on dairy farms is limited. Veterinarians serve as a key information source for US dairy producers with nearly 80% of dairy producers citing veterinarians as a very important source of information for making health care decisions for their operations (NAHMS, 1996). Furthermore, 98.1% of dairy producers surveyed used a veterinarian to diagnose or treat dairy animals on their operation in 1995 and, of those operations that used a veterinarian, 74.4% used their veterinarian for reproductive consultation/services (NAHMS, 1996). Because of their role in reproductive management decisions made on dairy farms, effective Extension efforts in dairy cattle reproduction must include veterinarians to effect widespread change in the dairy industry. On average, each bovine veterinarian in Wisconsin has the potential to impact 36 dairy producers (based on 18,000 dairy farms and approximately 500 dairy veterinarians). Although dairy producers can purchase their own ultrasound machine and conduct reproductive examinations on their own cows, they generally lack the knowledge, training, and experience needed to adequately perform such examinations. Furthermore, veterinarians are currently the only group who can legally perform reproductive ultrasound examinations for hire on dairy farms in Wisconsin and most other states in the United States.

## Bovine Reproductive Ultrasound Workshops

Development of Extension education programs to train bovine practitioners to use ultrasound for routine reproductive examinations is a critical step toward implementation of ultrasound technology into the dairy industry. In 1999, an Extension education workshop was developed and implemented in Wisconsin to train bovine practitioners in the practical applications of ultrasound for reproductive management of dairy cattle. The structure of the workshop provides timely, research-based reproductive information and practical hands-on ultrasound experience in an integrated manner.

To date, veterinarians from 20 different US states and 2 Canadian provinces have attended the Bovine Reproductive Ultrasound Workshops held in Wisconsin. That veterinarians travel from across the United States and Canada to attend these workshops in Wisconsin underscores the widespread demand for such training and the lack of adequate training opportunities in other regions of the country. On-farm Extension programs that bring the university together with veterinarians and individual farm owners and operators provide the structure from which significant change in the dairy industry can occur. The concept behind the Bovine Reproductive Ultrasound Workshops draws from the fundamental basis upon which university Extension and the Land Grant System were founded: to enhance the application of useful and practical information beyond the university campus through cooperative Extension efforts with states and local communities (NASULG, 1995).

## CONCLUSION

As a research tool, transrectal ultrasound has revolutionized our understanding of reproductive biology. As a management tool, transrectal ultrasound may provide a diagnostic tool for improving reproductive management in dairy operations. Although there are many potential applications of ultrasound for use in reproductive management of dairy cattle, combining ultrasound for early pregnancy diagnosis with timed AI along with early detection of twin pregnancies will likely result in the most widespread uses of this technology. Development of integrated reproductive management systems that combine ultrasound with new and existing reproductive technologies will further enhance the practical applications of ultrasonography. Further research on the efficacy of protocols that integrate ultrasonography with timed AI protocols for resynchronzation of ovulation, differential management strategies for cows carrying twin fetuses, and thorough economic analyses on the use of ultrasound for reproductive management of dairy cattle must be conducted before widespread integration of ultrasound occurs in the dairy industry.

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| Application Number: | 09/879,480 |
| Filed: | June 12, 2001 |
| Title: | Integrated Herd Management System Utilizing Isolated Populations of X-Chromosome Bearing and Y-Chromosome Bearing Spermatozoa |

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# Exhibit B

*IVF/IVP*

## 258 ULTRASTRUCTURAL STUDY OF STALLION SPERM FOLLOWING THAWING, CAPACITATING AND IVF PROCEDURES: EFFECT OF THE PRESENCE OF CUMULUS-ENCLOSED OOCYTES

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The use of IVF in horses has a limited efficiency, reflecting low oocyte developmental competence and inadequate sperm capacitation procedures. In a preliminary study, using carboxyfluorescein diacetate/propidium iodide staining, we determined that the freezing-thawing procedure left only 56.6 ± 3.4 % of the sperm cells with an intact membrane. The following incubation in TALP-IVF induced membrane damage at high rates with only 9.58 ± 1.8 % of them intact after 18 h. However, the presence of at least four cumulus-enclosed oocytes (CEO) in the medium significantly increased the number of membrane-intact spermatozoa at the end of incubation (53.87 ± 1.99%). This indicated that the sperm thawing and capacitating procedures can damage the cell membrane but the presence of four or more CEO in TALP-IVF could prevent further damages. The aim of the study was to investigate in detail the membrane damages and to analyze the differences induced by the presence of CEO. Spermatozoa were thawed in water at 37°C, and centrifuged for 30 minutes at 600*g* in a 45–90% Percoll gradient made with modified Tyrode's medium. The sperm pellet was washed once in the same medium and diluted to a final concentration of $1 \times 10^6$ spermatozoa/ml TALP supplemented with 0.6% (w/v) BSA fatty acid free and 12 $\mu g\,mL^{-1}$ heparin (TALP-IVF). Sperm cells were incubated with 0 or 4 in vitro-matured CEO. Sperm cells were examined after thawing, 0, 2 and 18 h from the beginning of incubation in TALP-IVF. Each experiment was replicated at least 3 times. Both scanning and transmission electron microscopy were performed on sperm samples fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, using standard procedures. Specimens for scanning electron microscopy were examined under a field emission gun JEOL JSM 6301 microscope. For transmission electron microscopy the samples were examined with a JEOL JEM 100 SX. A minimum of 25 cells were analyzed for each group. Immediately after thawing, damaged spermatozoa showed, on the surface of their heads, small vesicles correlated to a progressive process of vacuolisation and degeneration of membrane integrity. The same lesions were visible at all the successive time points taken into account. Moreover, a loss of the acrosome integrity with acrosomal swelling and a decrease of content homogeneity were observed particularly in the spermatozoa cultured for 18 h without CEO. When CEO were present in the IVF medium lesions were visible in a lower percentage of spermatozoa but the type of lesions did not differ from those observed in their absence. These observations confirmed our previous data and gave more details on the lesions that occur during the IVF procedures in the horse. Supported by MURST COFIN grant n. 2001078849.

## 259 PRODUCTION OF TROPICAL DAIRY CALVES BY EMBRYO TRANSFER USING LOCAL LAISIND (*BOS INDICUS*) RECIPIENTS AND INTERCONTINENTAL FRESH IVF SHIPPED EMBRYOS

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Increasing the diary population and milk production is a goal of many tropical developing countries. We report in this paper an attempt to develop a system of intercontinental shipping for transfer of fresh crossbred *Bos taurus* × *Bos indicus* IVF embryos into local Laisind (*Bos indicus*) recipients as a way to produce tropical dairy calves with highly improved milk productivity. The production of embryos was done at BOMED, Inc (Madison, WI, USA). Oocytes collected from ovaries of Holstein (*Bos taurus*) at slaughter and semen from milking Gir (*Bos indicus*) were used for IVP. Cleaved embryos were selected for air shipping in portable incubators at Day 4 (Group 1), Day 3 (Group 2) or Day 2 (Group 3) after IVF. The duration of shipping varied from 60 to 65 h. Embryo transfer was done in Vietnam. Laisind cows (Yellow cattle × Red Sindhi) with body weight more then 280 kg and normal reproductive activity were selected for treatment of estrous synchronization with double 11-day interval injection of PG2α (Intervet, Boxmeer, The Netherlands) and single injection of eCG (SABC Vietnam) two days before the second injection of PG2α. Timing of injections was calculated according to the IVF schedule. Embryos collected from portable incubators were transferred to a $CO_2$ incubator for further culture at 39°C. Two experiments were carried out: (1) transfer of embryos without sexing; (2) transfer of embryos after biopsy and sex determination by PCR. In experiment 2, compact morulae or morula-blastocysts were selected for sex determination. Four to five blastomeres were aspirated from each embryo using a cutting pipette and an aspiration pipette of 30-μm diameter. PCR was done as previously described (Uoc *et al.*, 1999 J. Biology). After biopsy, embryos were kept in culture for one day to observe the development in vitro. Embryos developed to morula-blastocyst or hatching blastocyst at Day 7 or Day 8 after IVF were transferred nonsurgically to recipients with estrus detected in the period from 0 to 12 h before or after the starting IVF. Pregnancy was confirmed by rectal palpation 3 months after embryo transfer. The average rate of embryos developed into morula-blastocyst was more then 50% (Table 1) and there were no significant differences among different shipping groups. For experiment 2, more then 87 % of embryos biopsied developed in vitro to expanding and hatching embryos. The average rate of female embryos was 56.3%. The pregnancy rate at 3 months was more then 44% ($n = 188$). The first group of calves was born without unusual birthing problems. In conclusion, the system of embryo transfer using intercontinental shipping of fresh IVF embryos and local *Bos indicus* recipients can be applied for production of dairy calves. Supported by grant from the AIRE-Development agency.

**Table 1. Development in vitro of IVP embryos**

| Experiment | Exp-1. without biopsy & sexing No. embryos observed | No. morulae at Day 8 (%) | No. blastocysts at Day 8 (%) | Exp. 2 with biopsy & sexing No. embryos treated | No. developed after cuture (%) | % of female embryos |
|---|---|---|---|---|---|---|
| Day 4 embryos | 242 | 38 (15.7) | 99 (41.2) | 89 | 74 (83.2) | 57.6 |
| Day 3 Embryos | 393 | 98 (24.9) | 126 (32.3) | 69 | 59 (85.5) | 62.9 |
| Day 2 Embryos | 323 | 56 (17.3) | 142 (44.2) | 64 | 61 (95.3) | 51.56 |
| Average | 958 | 192 (20.0) | 367 (38.3) | 222 | 194 (87.4) | 56.3 |

## 260 PRELIMINARY RESULTS OF IN VITRO FERTILIZATION FOR THE ENDANGERED MURCIANO-LEVANTINA BOVINE BREED

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The Murciano-levantina cattle from the Spanish Southeast belong to a bovine endangered breed under the special protection of FAO (WWL-DAD:3)*. Their characteristics of rusticity, longevity, docility and disease resistance make the recovery of the breed interesting. The objective of the present work was to determine the efficiency of in vitro bovine embryo production using oocytes collected from postmortem ovaries fertilized with frozen-thawed sperm obtained from either commercial or Murciano-levantina bulls. Cumulus-oocyte complexes were matured for 24 h in TCM199 with 10% FBS, 2 mM L-glutamine, 0.2 mM sodium pyruvate, 0.3 $\mu g\,mL^{-1}$ LH and 5 $\mu g\,mL^{-1}$ FSH. Oocytes presumed matured were fertilized with Percoll-prepared sperm (750 000/mL) in IVF-TALP, as described by Parrish *et al.*, (1988 Biol. Reprod. 38, 1171–1180). Zygotes (at 18 h) or two-cell-stage embryos (at 48 h) were stained with Hoechst 33342 (10 $\mu g\,mL^{-1}$; 20 min) and assessed by means of epifluorescence microscopy. The experiment was replicated on 4 occasions. The results of penetration (PEN-%), average number of spermatozoa inside the oocyte (S/O), monospermy (MON-%), putative embryos (zygotes) with two pronuclei (2 PN-%) and percentages of cleavage at 48 hours were recorded (Table 1). The results show that early embryos can be obtained with no differences in monospermy and cleavage rates regardless whether the sperm source was commercial or Murciano-levantina ($P \geq 0.05$).

*Lista Mundial de Vigilancia para la Diversidad de los Animales Domésticos (1997). http://www.fao.org/docrep/V8300S/V8300S00.htm.

**Table 1. In vitro fertilization results with one commercial and one Murciano-levantina bull**

| | $n^a$ | PEN[a] | S/O[a] | MON[a] | 2 PN[a] | $n^b$ | 1 CELL[b] | 2–4 CELLS[b] | >4 CELLS[b] |
|---|---|---|---|---|---|---|---|---|---|
| Commercial | 79 | 62.02 ± 5.49 | 1.38 ± 0.11 | 77.55 ± 6.02 | 61.22 ± 7.03 | 92 | 55.43 ± 5.21 | 34.78 ± 4.99 | 9.78 ± 3.11 |
| Murciano-levantina | 76 | 60.53 ± 5.64 | 1.48 ± 0.16 | 76.09 ± 6.35 | 73.91 ± 6.54 | 95 | 53.68 ± 5.14 | 29.47 ± 4.70 | 16.8 ± 3.85 |
| *P* | | 0.849 | 0.657 | 0.868 | 0.191 | | 0.811 | 0.440 | 0.158 |

[a]At 18 h. [b]At 48 h.

## 261 DIFFERENT TRANSVAGINAL OVUM PICK-UP STRATEGIES TO OPTIMIZE THE OOCYTE RETRIEVAL AND EMBRYO PRODUCTION OVER A FIXED PERIOD OF TIME

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The objective of this study was to compare the efficacy of different oocyte retrieval schemes over a period of 10 weeks. Fifteen multiparous Angus cows were randomly assigned ($n = 3$/group) to the following groups: 1) OPU once/week (7-day interval), 2) OPU twice/week (3- and 4-day interval, alternately), 3) Dominant follicle removal (DFR) + OPU once/week. DFR followed by OPU 72 h later, 4) DFR + FSH + OPU once/week. DFR followed 36 h later by FSH (Folltropin, Bioniche, Belleville, Ontario, Canada) (120 mg s.c. and 80 mg i.m. administered simultaneously) followed by OPU 48 h later, 5) FSH + OPU twice/week. FSH followed by $OPU_1$ 30 h later and $OPU_2$ 96 h after $OPU_1$. The interval between $OPU_2$ and next FSH was approximately 42 h. The follicles were aspirated using an Aloka ultrasound scanner (Wallingford, CT, USA) and a 5-MHz probe. The COCs were selected based on morphology and matured in TCM-199, supplemented with 10% FCS, 0.01 units $mL^{-1}$ bFSH, 0.01 units $mL^{-1}$ bLH and antibiotics. Fertilization (Day 0) was carried out with TALP-FERT medium containing capacitation factors. Frozen semen from the same bull was used ($1 \times 10^6\,mL^{-1}$) throughout. After 18 h the presumptive zygotes were cultured in SOF with 5% FCS (Holm P *et al.*, 1999 Theriogenology 52, 683–700). The embryos were evaluated based on IETS guidelines (Grades 1 and 2 selected). The data were analyzed by chi-square test and ANOVA. In all parameters, the DFR followed by FSH and subsequent OPU once/week protocol gave the best results on a per-cow-per-week as well as total (3 cows over 10 weeks) basis. Though OPU was done only once/week, this group produced more total oocytes (303) than groups where OPU was done twice/week, either with FSH (286) or without FSH (229) and also produced more total embryos on Day 8 (71 blastocysts, 23.4% of oocytes

cultured) than the latter two groups (64, 22.4% and 49, 21.4%, respectively). Among the nonstimulated groups, the OPU twice/week group had more total oocytes (236) than groups with OPU once/week, either without DFR (137) or with preceding DFR (160). However, a preceding DFR seemed to have a positive effect on oocyte quality as this group had a better embryo development rate (26.9%), producing more total embryos (42). In comparison, OPU twice/week produced total 49 embryos (21.4%) and OPU once/week produced 26 (19.4%). In conclusion, DFR coupled with single-shot FSH administration can be used effectively over a period of at least 10 consecutive weeks and can increase ($P < 0.05$) the oocyte yield by two-fold and embryo production following IVF by two and half-fold, as compared to routine OPU-IVF done once a week.

**Table 1. Per cow per week performance**

| Treatment groups | Follicles aspirated | Oocytes retrieved | Oocytes cultured | Blastocysts Day 7 | Blastocysts Days (7 + 8) | Embryos hatched |
|---|---|---|---|---|---|---|
| OPU 1/w | $7.8 \pm 2.4^{a}$ | $4.6 \pm 1.9^{a}$ | $4.5 \pm 1.9^{a}$ | $0.6 \pm 0.8^{a}$ | $0.9 \pm 0.9^{a}$ | $0.2 \pm 0.4^{a}$ |
| OPU 2/w* | $13.0 \pm 3.5^{b}$ | $7.9 \pm 2.9^{b}$ | $7.6 \pm 2.8^{b}$ | $1.3 \pm 1.1^{bc}$ | $1.6 \pm 1.3^{bc}$ | $0.5 \pm 0.9^{ab}$ |
| DFR-OPU 1/w | $7.3 \pm 2.4^{a}$ | $5.3 \pm 2.2^{a}$ | $5.2 \pm 2.1^{a}$ | $1.2 \pm 1.3^{ab}$ | $1.4 \pm 1.6^{ab}$ | $0.5 \pm 0.8^{ac}$ |
| DFR-FSH-OPU 1/w | $16.0 \pm 5.0^{c}$ | $10.6 \pm 4.5^{c}$ | $10.1 \pm 4.4^{c}$ | $2.1 \pm 1.2^{d}$ | $2.4 \pm 1.4^{d}$ | $0.7 \pm 0.9^{bc}$ |
| FSH-OPU 2/w* | $15.1 \pm 4.4^{c}$ | $9.8 \pm 3.9^{c}$ | $9.5 \pm 3.8^{c}$ | $1.8 \pm 1.6^{cd}$ | $2.1 \pm 1.9^{cd}$ | $0.7 \pm 0.8^{bc}$ |

Values not having a common superscript in the same column differ ($P < 0.05$). *2 sessions/week.

## 262 IN VITRO FERTILIZATION IN MICROFLUIDIC CHANNELS ENHANCES MONOSPERMIC PENETRATION OF SWINE OOCYTES

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In traditional porcine IVF systems, there is a high incidence of polyspermy, a pathological condition which results in aberrant embryonic development. Efforts to improve the in vitro embryo production process in pigs have included modifying the culture medium, the number of spermatozoa inseminated, and the quantity of medium used. Recently, the development of microscale embryo culture devices (microchannels) has opened new avenues for manipulation of the IVF system to improve the efficiency and overall production of porcine embryos by more closely mimicking the function of the oviduct. The volume of medium in the local vicinity of the embryo is smaller (0.125 μL) compared to the typical 5–50 μL microdrops. Additionally, it is believed that the delivery of sperm cells in the microchannel simulates the flow pattern of spermatozoa past the oocytes similar to that in the oviduct. This study was designed to compare the incidence of polyspermy of pig oocytes fertilized in PDMS-glass microchannels (MC) to conventional microdrop methods (controls). Oocytes were obtained by aspiration of ovarian antral follicles. Fifty oocytes were placed into 500 μL of TCM199 medium supplemented with LH, FSH, EGF, cysteine, PVA, and antibiotics. Extended semen was washed with mTBM and re-suspended to $6 \times 10^5$ sperm/mL. The sperm suspension was placed in humidified 5% $CO_2$ in air atmosphere at 39°C for 1 h to allow for capacitation. Concurrently, pre-warmed microchannels were filled with 200 μL of mTBM and allowed to equilibrate for 1 h at 39°C. Cumulus cells were removed from the oocytes using 0.1 mg $mL^{-1}$ hyaluronidase in mTBM. At 44 h of maturation, 15 oocytes were placed into a pre-equilibrated 50 μL drop of mTBM covered with warm paraffin oil in a petri dish, and placed into the microchannel. Capacitated sperm cells were then added to the oocytes to give a final concentration of $3 \times 10^5$ sperm/mL; the mixture was incubated for 6 h. Presumptive zygotes were cultured in 100 μL drops of NCSU-23 covered with oil at 39°C in a humidified 5% $CO_2$ in air for 12 h. At this time, the zygotes were fixed in 1 : 3 (v/v) glacial acetic acid in absolute ethanol for 48–72 h. Aceto-orcein staining data revealed a higher incidence of monospermic penetration and a lower number of spermatozoa per oocyte in the microchannels as compared to the controls (Table 1). Data from six replicates were arranged in a randomized block design and analyzed by the generalized linear model in SAS. These data support the idea that the microchannel environment reduces the incidence of polyspermy during IVF of porcine oocytes ($P < 0.05$) while maintaining comparable penetration and male pronuclear formation rates. Furthermore, it is possible that the number of sperm present near the oocytes during fertilization is decreased using the microchannel. In conclusion, microfluidic technology has shown the potential to improve in vitro fertilization in swine by an increasing monospermic penetration of oocytes.

**Table 1. Effect of IVF system on fertilization parameters**

| IVF System | Oocyte number | Maturation (% of total) | Penetration (% of matured) | Monospermic (% of penetrated) | MPF (% of penetrated) | No. Sperm/ oocyte |
|---|---|---|---|---|---|---|
| Control | 138 | $88.59 \pm 1.1$ | $95.71 \pm 0.8$ | $20.56 \pm 1.6^{a}$ | $77.84 \pm 1.7$ | $6.65 \pm 0.4^{a}$ |
| MC | 128 | $80.22 \pm 1.4$ | $86.60 \pm 1.6$ | $57.99 \pm 1.7^{b}$ | $70.76 \pm 2.1$ | $1.77 \pm 0.1^{b}$ |

[a,b]different superscripts within columns represent significant differences ($P < 0.05$). Values are mean ± SEM. MC: microchannel; MPF: male pronuclear formation.

## 263 BINDING OF STALLION SPERM TO EQUINE AND BOVINE ZONAE PELLUCIDAE: EFFECT OF MILK, MILK PROTEINS AND GLUCOSE

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The ability of sperm to bind to zonae pellucidae (ZP) has been correlated with fertilizing capacity of sperm in several species. Limited numbers of equine oocytes are available to perform such assays. Therefore, use of heterologous ZP to perform gamete binding tests with stallion sperm would be useful. We have found that addition of 10% of skim milk-based extender with glucose [EZ-Mixin®, Animal Reproduction Systems, Chino, USA; (EZ)] to TALP significantly increased the number of stallion sperm bound to bovine ZP. Objectives of the present experiments were to determine: (1) if stallion sperm bind in similar numbers to equine and bovine ZP, and (2) the effects of skim milk, milk proteins and glucose on sperm binding to ZP. Denuded bovine (immature) and equine (mature) oocytes were stored at 5°C in salt solution (1.5 M $MgCl_2$, 40 mM HEPES, 0.1% PVP). In Experiment I, 4 ejaculates from 2 stallions were centrifuged at 300*g* for 6 min, and sperm pellets were resuspended in 1 mL of TALP or EZ. Sperm were stained with Hoechst 33342, centrifuged, and resuspended to $2 \times 10^6$ sperm $mL^{-1}$. Oocytes were placed into droplets of 45 μL of TALP (7 to 10 oocytes/trt/ejac). Extended sperm (5 μL) were added to oocytes, resulting in $2 \times 10^5$ sperm $mL^{-1}$, and the mixutre was incubated for 2 h at 38.5°C. Oocytes then were pipetted in TALP to remove loosely attached sperm and observed with fluorescence microscopy; mean numbers of sperm bound to bovine and equine ZP for TALP were $29 \pm 1.9$ and $36 \pm 2.6$ ($P > 0.1$) and for EZ, $149 \pm 5$ and $152 \pm 6.3$ ($P > 0.1$), respectively. More sperm bound to ZP with EZ than to ZP with TALP ($P < 0.001$). Experiment II used 4 ejaculates from 4 stallions. After initial centrifugation, sperm were resuspended in 1 mL of each of six extenders: TALP, EZ, TALP containing 89.5 mM glucose (TG), TALP containing 163.5 mM glucose (THG), TALP containing 2.4 mg $mL^{-1}$ of skim-milk powder (TSM), and INRA 96® (IMV Technologies, L'Aigle, France) that contains 27 mg $mL^{-1}$ of native phosphocaseinate. Hoechst 33342-stained sperm and bovine oocytes were processed as described for Experiment I. Treatments containing milk proteins resulted in more sperm binding ($P < 0.01$) than those without milk proteins (Table 1). In conclusion, use of bovine oocytes led to similar results for equine and bovine oocytes; therefore, bovine oocytes can be used for binding assays with stallion sperm. High concentrations of glucose increased numbers of sperm bound to ZP; however, presence of milk or milk proteins was more effective in enhancing binding of sperm to ZP. INRA96 contains relatively low glucose (67 mM) and one milk protein. Therefore, we hypothesize that native phosphocaseinate may cause increased sperm binding to ZP.

**Table 1. Mean sperm bound per ZP ± SEM ($n = 38$–40/group)**

| Treatment | TALP | TG | THG | TSM | EZ | INRA 96 |
|---|---|---|---|---|---|---|
| Sperm/ZP | $53 \pm 3.9^a$ | $55 \pm 3.6^a$ | $81 \pm 4.3^b$ | $169 \pm 5.3^c$ | $160 \pm 5.5^c$ | $151 \pm 5.2^c$ |

[abc]Means with different superscripts differ significantly, ($P < 0.01$); ANOVA of log transformed data.

## 264 IN VITRO FERTILIZATION OF *MACACA NEMESTRINA* OOCYTES WITH FRESH AND FROZEN-THAWED EPIDIDYMAL SPERM

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In an effort to develop methods to maximize the use of reproductive tissues in our own tissue distribution program (WaNPRC TDP) and to serve as platforms for application of ART to endangered non-human primate (NHP) species, we have examined the effects of collection and cryostorage on epididymal sperm (ES) collected from *Macaca nemestrina* (Mn). Fresh ES was collected by needle aspiration from the cauda epididymis and prepared by centrifugation in mHTF (Irvine Scientific, Santa Ana, CA, USA) + BSA (3 mg $mL^{-1}$) at 700*g* for 10 min. The resultant pellet was resuspended in fresh mHTF + BSA and held at RT until required. ES subjected to freeze-thaw was collected from the cauda epididymis of testes provided by the WaNPRC TDP by slicing and swim-out into mHTF + BSA. ES was frozen in mTTE medium + 5% glycerol (Sankai T *et al.*, 1994 J. Reprod. Fertil. 101, 273). Frozen ES straws were removed from liquid nitrogen and thawed in air at RT for 10 min. Thawed ES was centrifuged through an 80% PureSperm gradient (Spectrum Technologies, Healdsburg, CA, USA) at 700 g for 15 min. The resultant pellet was retrieved and washed in mHTF + BSA at 600 g for 5 min. For capacitation sperm were incubated in pre-equilibrated HTF + BSA containing 1.0 mM caffeine and 0.1 mM dbcAMP (Sigma, St. Louis, MO, USA) for 5–10 min in a humidified atmosphere of 6% $CO_2$ in air. In vitro-matured Mn oocytes collected from unstimulated ovaries were exposed to fresh or frozen-thawed ES whereas in vivo-matured Mn oocytes collected from superovulated ovaries were exposed to fresh ES in 50-μL Fertilized oocytes were cultured for 24–48 h and assessed for cleavage on Day 3 post-insemination. Data were analyzed by single-factor ANOVA or *t*-test where appropriate and are expressed as mean ± SD. Fresh Mn ES ($n = 4$) exhibited very low motility at collection ($38 \pm 10\%$) but recovered motility following processing ($58 \pm 20\%$). Frozen-thawed ES ($n = 4$) also exhibited low motility post-thaw ($53 \pm 17\%$) and recovered after processing ($66 \pm 28\%$). The percentage of acrosome-intact sperm was not significantly different ($P > 0.05$) for fresh ES ($58.9 \pm 8.6\%$) compared to frozen-thawed ES ($70.6 \pm 21.9\%$). Fresh and frozen-thawed ES fertilized in vitro-matured Mn oocytes at similar rates (fresh $68 \pm 10.3\%$, $n = 170$ v. frozen-thawed $71.2 \pm 6.7\%$, $n = 90$; $P > 0.05$). Cleavage rates of fertilized IVM oocytes were not significantly different (fresh $79 \pm 7.2\%$ v. frozen-thawed $79.8 \pm 5.0\%$; $P > 0.05$). Fresh ES was also able to fertilize in vivo-matured Mn oocytes collected from superovulated ovaries ($95.1 \pm 2.5\%$, $n = 98$) and fertilized oocytes went on to cleave at a high rate ($96.1 \pm 2.6\%$). These results suggest that fresh and frozen-thawed ES may be useful for applied ART in endangered species. This work was supported by NIH grant #RR00166 and the WaNPRC TDP.

## 265 EFFECT OF SPECIFIC GRAVITY OF CULTURE MEDIUM ON IN VITRO EMBRYO DEVELOPMENT IN BUFFALO

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In buffalo the success rate of transferable quality embryo production through in vitro procedure is very low as compared to cattle. Sub optimal culture conditions and physical conditions such as specific gravity of the culture medium may lead to a reduced rate of transferable buffalo embryo production from the oocytes matured and fertilized in vitro (Palta & Chauhan,1998 Reprod. Fertil. Dev. 10, 379–391). This experiment was therefore conducted to find out the role of specific gravity of the IVC medium on the development rate of the buffalo embryos in vitro. Follicles of slaughter house ovaries were aspirated and the collected oocytes with cumulus-oocytes complexes (COCs) were cultured in TCM-199 medium supplemented with 10% fetal calf serum, 10% buffalo follicular fluid and 0.5 $\mu g\,mL^{-1}$ FSH in 5% $CO_2$ incubator at 38.5°C. The matured oocytes were then inseminated with frozen-thawed buffalo semen suspended in BO medium. After 42 h of post-inseminations the cleavage rates were evaluated. The 2–4 cell-cleaved eggs (Day 2 of post-insemination) were randomly divided and cultured for eight days in vitro in 1) modified synthetic fluid (mSOF) + 0.8 % BSA (control), 2) mSOF + 0.8 % BSA + gelatin (1 $mg\,mL^{-1}$) 3) mSOF + 0.8% BSA + 1 $mg\,mL^{-1}$ gelatin + 10 $ng\,mL^{-1}$ epidermal growth factor (EGF). Supplementation of gelatin increased the specific gravity of the mSOF medium from 0.9658 ± 0.009 to 1.0331 ± 0.013 without any change in pH (7.4). The development of embryos to the 8–16 cell-stage on day 4 of in vitro culture were significantly higher ($P < 0.05$) in mSOF + 0.8% BSA + 1 $mg\,mL^{-1}$ gelatin (81.8%; 27/33) than that in mSOF + 0.8% BSA (75.7%; 28/37) and mSOF + 0.8% BSA + 10 ng/mL EGF (68.7%; 22/32). When these embryos were further cultured for another four days (Day 8), the development of transferable quality embryos (morula/blastocyst) was 42.4% (14/33), 48.7% (18/37) and 46.9% (15/32), respectively. Supplementation of gelatin increased the cleavage of eggs up to the 8–16 cell-stage embryo, but did not significantly enhance the rate of development to the morula/blastocyst stage in comparison to control and EGF-supplemented group. However, the percentage of transferable quality embryos was slightly lower in the gelatin-added group but not statistically significant than other groups. The study concluded that increase in specific gravity of the in vitro culture medium enhanced initial cleavage rate but did not have any role in transferable embryo production in buffalo.

## 266 IN VITRO-DERIVED EMBRYO PRODUCTION WITH SEXED AND UNSEXED SEMEN FROM DIFFERENT BULLS

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The production of pre-sex-selected calves by in vitro fertilization (IVF), using sexed semen, does show some benefits due to the small quantity of sperms needed for the process as compared to other reproductive technologies. The objective of this study was to determine differences among bulls and sperm concentrations in embryo development with sexed and unsexed semen. Follicles ranging from 2 to 6 mm in diameter were aspirated from slaughterhouse ovaries. COC were selected and matured in groups of maximum of 30 in 1.8 mL of TCM-199, supplemented with 10% fetal calf serum, 0.01 $U\,mL^{-1}$ bFSH, 0.01 $U\,mL^{-1}$ bLH and 10 $\mu L\,mL^{-1}$ penicillin-streptomycin for 24 h at 38.5°C. Fertilization (Day 0) was carried out in micro-drops (50 $\mu L$) with TALP-FERT medium containing PHE (3 $\mu g\,mL^{-1}$ penicillamine, 11 $\mu g\,mL^{-1}$ hypotaurine and 0.18 $\mu g\,mL^{-1}$ epinephrine), 10 $\mu L\,mL^{-1}$ non-essential amino acid and 2 $\mu g\,mL^{-1}$ heparin. Frozen/thawed sexed (female) and non-sexed sperms from five bulls were selected in a discontinuous percoll gradient. Sperm concentration was $1 \times 10^6$ for non-sexed semen and $1 \times 10^6$ or $2 \times 10^6$ for sexed semen. After 18–20 h, presumptive zygotes were denuded and cultured in groups of 10 in 50-$\mu L$ micro-drops of SOF citrate with 5% FCS (Holm P *et al.*, 1999 Theriogenology 52, 683–700) under paraffin oil in a 5% $O_2$, 5% $CO_2$, 90% $N_2$ atmosphere with high humidity. On Day 7, blastocysts (BL) were morphologically evaluated and recorded. Results are shown in Table 1. Data was compared by chi-square analysis. Sexed frozen bovine sperm can be used successfully in IVF systems. More research needs to be done to optimize and standardize bovine in vitro fertilization with sexed semen.

**Table 1. Results of comparisons between bulls, sperm concentrations, cleavage and embryo development**

| Bulls | Sperm Concentration | Semen | Cleavage (%) | Development to BL stage on Day 7 (%) |
|---|---|---|---|---|
| Holstein #1 | $1 \times 10^6$ | Sexed | 65/225 (29%)[a] | 8/225 (3.5%)[a] |
| Holstein #1 | $2 \times 10^6$ | Sexed | 62/196 (31.6%)[a] | 11/196 (5.6%)[a] |
| Holstein #1 | $1 \times 10^6$ | Unsexed | 366/474 (77%)[b] | 127/474 (26.8%)[b] |
| Holstein #2 | $1 \times 10^6$ | Sexed | 78/321 (24%)[*] | 16/321 (5%)[*] |
| Holstein #2 | $2 \times 10^6$ | Sexed | 116/215 (54%)[**] | 30/215 (14%)[**] |
| Holstein #2 | $1 \times 10^6$ | Unsexed | 336/422 (79.6%)[***] | 125/422 (29.6)[***] |
| Holstein #3 | $2 \times 10^6$ | Sexed | – | 26/118 (22%) |
| Holstein #3 | $1 \times 10^6$ | Unsexed | – | 17/108 (15.7%) |
| Jersey #1 | $2 \times 10^6$ | Sexed | – | 25/115 (21.7%) |
| Jersey #1 | $1 \times 10^6$ | Unsexed | – | 22/65 (33.8%) |
| Jersey #2 | $2 \times 10^6$ | Sexed | – | 27/148 (18%) |
| Jersey #2 | $1 \times 10^6$ | Unsexed | – | 17/142 (12%) |

Value with different superscripts in the same column differ ($P < 0.05$).

## 267 BODY WEIGHT GAIN VARIATION, HORMONAL AND METABOLIC STATUS AND IN VITRO EMBRYO PRODUCTION IN SUPEROVULATED DAIRY HEIFERS

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This study aimed to investigate the hormonal and metabolic status of heifers subjected to short-term variation of energy intake and associated growth rate; oocytes were collected by ovum pick-up (OPU) for in vitro embryo production. Experimental scheme, diets and OPU protocol have been described previously (Freret *et al.*, 2003 Theriogenology, 59, 445 abst). Briefly, oocytes from 16 Prim'Holstein heifers (14 ± 1 months old, 340 ± 25 kg) were collected by OPU every two weeks after superovulation treatment (total dose of 250 μg FSH (Stimufol®, Merial, France) divided into 5 i.m. injections 12 hours apart, at decreasing doses). They received individually for 6 weeks (Period 1 = OPU 1 to 4) a diet aimed at a 1000 g $day^{-1}$ body weight gain (BWG). Heifers were then allocated to 2 groups (overfeeding or dietary restriction), for 8 weeks after OPU 4 (Period 2 = OPU 5 to 8). COCs were collected 12 h after the last FSH injection for IVF and IVC. Blood sample analyses were performed once a week to determine glucose, insulin, IGF1, non esterified fatty acids (NEFA), β-hydroxybutyrate (βOH) and urea concentrations, and at the time of follicular puncture for estradiol assay. Effects of period, group of growth rate and their interaction where analyzed using the mixed procedure of SAS (female effect as random) and least-squares means were subsequently compared with Scheffe's test. Three groups of growth rate were determined according to results observed during period 2 (Table 1). A period effect was observed for glucose, insuline and estradiol ($P < 0.05$). But Scheffe's test showed a significant variation between periods only in the '600 g $day^{-1}$' group, with more estradiol and less glucose in period 2 which was associated with more blastocysts and grade 1 embryos (Freret *et al.*, 2003 Theriogenology, 59, 445 abst), and in the '1400 g $day^{-1}$' group with more insulin in period 2 (associated with more follicles <8 mm 2 days before FSH treatment). In period 2, βOH concentration was significantly higher in the '1400 g $day^{-1}$' group when compared to the others (Table 1). For urea, NEFA and IGF1 concentrations, no difference between groups or periods was observed. These results illustrate the role of glucose and insulin as mediators of nutritional effects on reproduction in growing animals. These results suggest that nutritional requirements aimed at optimizing follicular growth and embryonic development may be different.

**Table 1.**

| | Group 600 g $day^{-1}$ ($n = 4$) | | Group 1000 g $day^{-1}$ ($n = 8$) | | Group 1400 g $day^{-1}$ ($n = 4$) | |
|---|---|---|---|---|---|---|
| | Period 1 | Period 2 | Period 1 | Period 2 | Period 1 | Period 2 |
| BWG (g $day^{-1}$) | 940 ± 145 | 615 ± 100[a] | 980 ± 120 | 1035 ± 70 | 890 ± 200 | 1380 ± 100[c] |
| Follicles <8 mm 2 days before FSH | 27.2 ± 4.4 | 23.4 ± 4.4 | 22.2 ± 3.1 | 25.3 ± 3.1 | 19.1 ± 4.4[a] | 28.8 ± 4.4[b] |
| Estradiol at OPU (pg $mL^{-1}$) | 4.6 ± 2.14[a] | 12.01 ± 2.14[c] | 8.27 ± 1.51 | 10.1 ± 1.51 | 10 ± 2.14 | 11.68 ± 2.14 |
| Glucose (mmol $L^{-1}$) | 5.01 ± 0.13[a] | 4.67 ± 0.13[b] | 4.91 ± 0.09 | 4.76 ± 0.09 | 5.08 ± 0.13 | 5.02 ± 0.13 |
| Insulin (pmol $L^{-1}$) | 128.32 ± 19.6 | 156.12 ± 20.39 | 136.17 ± 13.86 | 147.56 ± 14.12 | 138.4 ± 19.6[a] | 207.4 ± 19.6[c] |
| βOH (mmol $L^{-1}$) | 0.35 ± 0.14 | 0.35 ± 0.14[c] | 0.73 ± 0.1 | 0.69 ± 0.1[b] | 1.12 ± 0.14 | 1.27 ± 0.14[a] |

Least-squares means ± SEM, [a v. b]: $P < 0.05$; [a v. c]: $P < 0.01$.

## 268 PREGNANCIES FROM FROZEN IVF CATTLE EMBRYOS USING SEX-SORTED AND UNSORTED SPERM

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Previously we demonstrated that sex-sorted sperm could produce IVF embryos from juvenile and adult cattle at rates similar to those for unsorted sperm (Fry *et al.*, 2003 Theriogenology 52, 198). In this study we investigated the pregnancy rates of recipient cattle following the transfer of frozen/thawed IVF embryos generated from young heifers using sex-sorted and unsorted sperm. COCs collected from FSH-stimulated Senepol or Beefex heifers by TVR were matured, fertilized with either sex-sorted or unsorted Senepol sperm and cultured for 6 days under our standard laboratory conditions (Fry *et al.*, 2003 Theriogenology 59, 446, Earl *et al.*, 1997 Theriogenology 47, 255). Embryos reaching the blastocyst or expanded blastocyst stage of development were frozen by the CL-V method of vitrification. Briefly, embryos were equilibrated for 5–10 min in HEPES-199 media containing 20% FCS (HM), placed in HM containing 10% EG, 10% DMSO for approximately 2 minutes and then in HM containing 20% EG, 20% DMSO for between 20–60 sec (Vatja *et al.*, 1997 Cryoletters 18, 191). Vitrification was achieved by collecting between 5–10 IVF embryos in a 3-μL droplet and securing this droplet to a coded CL-V holder. The droplet was vitrified using the CL-V kit (Lindemans *et al.*, 2004 Theriogenology in press) and then sealed in a precooled "straw" for storage in liquid nitrogen. To thaw, the "straw" with specimen was removed from storage; the specimen droplet was withdrawn from the "straw" and placed directly into HM containg 0.2 M sucrose (SM). After approximately 5–10 min each embryo was assessed, loaded into a tomcat catheter in SM and transferred surgically into a recipeint cow within 10–15 min of thaw. Of 129 Brahman and Brahman cross cows receiving 2 injections of 125 μg cloprostenol 11 days apart, 60 exhibited oestrus 2–4 days after the second

injection and 53 were deemed suitable for embryo transfer. Pregnancy was determined by ultrasound on Day 40. No difference in pregnancy rate was found between treatment groups ($P > 0.05$; Table 1). The low submission rate (60/129) and pregnancy rate for the in vivo control group indicate that the fertility of the recipient cows may have been compromised by the drought conditions predominating in Central Queensland. Notwithstanding, the CL-V method for the vitrification of IVF embryos produced by either sex-sorted or unsorted sperm gave similar and very promising pregnancy results of around 40%. This provides new opportunities for the rapid banking of large numbers of sexed IVF embryos generated from elite cattle by TVR for user friendly embryo transfer programs.

**Table 1. Pregnancies from IVF embryos derived from sex-sorted and unsorted sperm and frozen by the CL-V method of vitrification, or from in vivo embryos frozen in glycerol**

| Embryos | Sex-sorted IVF | Unsorted IVF | In vivo |
|---|---|---|---|
| Grade 1 | 3/6 | 8/17 | 3/8 |
| Grade 2 | 2/7 | 1/6 | 0/0 |
| Grade 3 | 3/7 | 0/1 | 0/1 |
| TOTAL | 8/20 (40%) | 9/24 (38%) | 3/9 (33%) |

## 269 IN VITRO FERTILITY OF BOAR SPERMATOZOA PRESERVED AT 10°C FOR 22 DAYS

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Fertility of boar spermatozoa as determined following artificial insemination seems to be maintained during liquid preservation at 10–15°C for several days, although prolonged liquid preservations reduce the pregnancy rate rapidly. However, it is not clear if spermatozoa can penetrate into oocytes in an IVF system even after a prolonged liquid preservation. Oxidative stress could also be one of the possible detrimental factors in liquid preservation of spermatozoa. In the present study, fertility of liquid-preserved spermatozoa was examined using an IVM-IVF system. Whether cysteine can improve the fertility was also determined. Spermatozoa (from four Berkshires) was resuspended at $1 \times 10^8$ cells $mL^{-1}$ in Modena solution containing 15% (v/v) boar seminal plasma and 0 or 5 mM cysteine after washing 3 times. Sperm suspensions (1 mL) were then preserved at 10°C for 22 days following a program for cooling down (to 15°C for 4 h, keeping at 15°C for 12 h and then to 10°C for 6 h). At Days 1, 8, 15 and 22 after the start of preservation, spermatozoa ($5 \times 10^5$ cells $mL^{-1}$) were co-cultured with IVM oocytes in an IVM/IVF system (Funahashi *et al.*, 1997 Biol Reprod 57, 49–53). Viability and functional status of spermatozoa were also examined at Days 8 and 15 of preservation by using LIVE/DEAD sperm viability kit and CTC fluorescence assay. Data (mean ± SEM) from 4–6 replicates were analyzed by ANOVA and Fisher's protected LSD test. When spermatozoa that had been preserved without cysteine (Cys−) were used, penetration rates were not different ($P > 0.05$) from those with cysteine (Cys+) at Day 8 of preservation (91.4 ± 3.4% in Cys− and 99.3 ± 0.7% in Cys+), but lower ($P < 0.02$) at Days 15 and 22 (72.6 ± 13.6% and 33.8 ± 8.4% in Cys−; 94.8 ± 2.1% and 71.1 ± 10.8% in Cys+, respectively). Both viability and proportion of uncapacitated live cells were higher ($P < 0.05$) in Cys+ than Cys− at Days 8 and 15. These results demonstrate that boar spermatozoa can penetrate into oocytes in vitro even after a liquid preservation at 10°C for 22 days and that cysteine can improve the viability and penetrability in vitro of spermatozoa during liquid preservation. Supported by the Ito Foundation.

## 270 IN VITRO FERTILIZATION OF BUFFALO (*BUBALUS BUBALIS*) OOCYTES: EFFECTS OF MEDIA AND SPERM MOTILITY INDUCING AGENTS

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The identification of an optimal in vitro fertilization system is critical in order to improve the in vitro embryo production efficiency in buffalo species. The aim of this work was to evaluate the effects of fertilization media and sperm motility inducing factors (SMIF) on cleavage and blastocyst rates in buffalo species. Cumulus-oocytes complexes ($n = 516$), recovered from slaughtered animals, were matured in vitro in TCM 199 + 10 % FCS, 0.5 $\mu g\,mL^{-1}$ FSH, 5 $\mu g\,mL^{-1}$ LH, 1 $\mu g\,mL^{-1}$ 17β-estradiol and 50 $\mu M$ cysteamine, at 38.5°C under 5% $CO_2$ in humidified air for 24 hours. The mature oocytes were randomly assigned to four groups for fertilization. In particular, IVF was carried out at 38.5°C under 5% $CO_2$ in humidified air in either Tyrode's modified medium or Brackett Oliphant medium, in the presence of 0.01 mM heparin; each medium was supplemented with either a mixture of 0.2 mM penicillamine and 0.1 mM hypotaurine or 5 mM caffeine. Frozen-thawed sperm from a tested bull was treated by the swim-up procedure and used at a final concentration of $20^6$ $mL^{-1}$. After 20–22 h presumptive zygotes were cultured in SOF medium, supplemented with essential and non-essential amino acids and BSA, in a gas atmosphere of 5% $CO_2$, 7% $O_2$ , and 88% $N_2$, up to the blastocyst stage. Cleavage rates and blastocyst yields were analyzed by a full factorial model 2 × 2 with medium and SMIF effects (SPSS 11.0). The analysis used permits the identification of statistical differences between treatments irrespective of an interaction (Searle SR. 1971. Linear model. Ed. John Wiley & Sons; XXI:533). The comparison of the two media, irrespective of the SMIF used, did not show any difference in cleavage rate (43.7% v. 39.3%, respectively, in TALP and BO). On the contrary higher cleavage rates were recorded with hypotaurine-penicillamine v. caffeine (47.7% v. 35.3%; $P < 0.05$), regardless of the medium employed. However, a significant interaction between media and SMIF was found; in fact the addition of hypotaurine and

penicillamine significantly improved cleavage rate compared with caffeine in TALP medium (59.6% v. 27.9%; $P < 0.05$) whereas no differences were observed in BO (35.9% v. 42.7%, respectively). With regard to blastocyst yield a significant effect of medium was also found, with the highest embryo production in TALP v. BO (13.9% v. 6.8%; $P < 0.05$). Blastocyst rate was improved in the presence of hypotaurine-penicillamine v. caffeine (13.6% v. 7.2%; $P < 0.05$). Furthermore there was a significant interaction between medium and SMIF, with the highest embryo yields in the presence of hypotaurine-penicillamine v. caffeine in TALP (20.7% v. 7.1%, respectively; $P < 0.05$) but not in BO (6.4% v. 7.2%, respectively). The differences we found disappeared when the embryo yield was calculated in relation to the cleaved eggs, with the exception of a lower efficiency of BO v. TALP (15.9% v. 31.1%; $P = 0.057$), suggesting an influence of BO also on post-fertilization development.

## 271 INFLUENCE OF ARGININE–GLYCINE–ASPARTIC ACID (RGD) IN BOVINE SPERM-EGG BINDING, AND FERTILIZATION IN VITRO

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Osteopontin (OPN), a secretory RGD-containing phosphoprotein, has been identified in cow oviductal ephitelium and fluid, but its role in fertilization is unclear. RGD peptide is capable of blocking fertilization, inducing intracellular $Ca^{2+}$ transients, and initiating parthenogenetic development when present during bovine fertilization in vitro.This study was conducted to determine whether in vitro sperm binding to the zona pellucida (ZP) and fertilization of bovine oocytes were affected by treating the sperm or oocytes with RGD (arginine–glycine–aspartic acid, a sequence recognized by integrins) or non-RGD-containing peptides. In vitro matured oocytes were incubated (39°C, 5% $CO_2$ in air) for 2 hours in fertilization medium with: (1) no peptides; (2) 50 $\mu g\,mL^{-1}$ RGD (Calbiochem®, San Diego, CA, USA); (3) 1000 $\mu g\,mL^{-1}$; (4) 50 $\mu g\,mL^{-1}$ non-RGD (Calbiochem®); (5) 1000 $\mu g\,mL^{-1}$ non-RGD. The bovine sperm from two differents bulle was collected by artificial vagina, pooled, washed twice with MTM at 500 g for 10 min and incubated (39°C, 5% $CO_2$ in air) for two h at $5 \times 10^7$ concentration in MTM with: (6) no peptides; (7) 50 $\mu g\,mL^{-1}$ RGD; (8) 1000 $\mu g\,mL^{-1}$; (9) 50 $\mu g\,mL^{-1}$ non-RGD; (10) 1000 $\mu g\,mL^{-1}$ non-RGD. Following incubation, treated and untreated oocytes were washed and inseminated with $1 \times 10^5$ treated or untreated fresh spermatozoa per 10 oocytes; after the sperm were recovered from a Percoll gradient (45%/90%). After 18–20 h, oocytes were removed from co-culture, and washed in TL-HEPES. Oocytes used to assess sperm binding were stained with Hoescht 33342, and the number of sperm bound per ZP counted. The remaining oocytes were fixed in acid alcohol, stained with 1% acetato-orcein and observed for the presence of pronuclei. For the five replicates, 100–120 oocytes were used for fertilization and 150–170 oocytes were used for sperm-egg binding assays. Data were analized by SAS. Treatment of sperm or oocytes with the RGD peptide significantly decreased ($P < 0.05$) fertilization compared to the non-treated controls or those treated with non-RGD peptides: (1) 80% ± 3.0; (2) 42% ± 3.0; (3) 30.2% ± 3.0; (4) 78.5% ± 3.0; (5) 79.1% ± 3.0; (6) 78.9% ± 3.0; (7) 41.3% ± 3.0; (8) 29.1% ± 3.0; (9) 79.2% ± 3.0; (10) 80.2% ± 3.0. More sperm bound to the ZP of untreated or non-RGD-treated oocytes or sperm than those incubated with the RGD peptide: (1) 71.2 ± 4.1; (2) 33.2 ± 4.2; (3) 24.2 ± 4.1; (4) 69.5 ± 4.1; (5) 70.2 ± 4.2; (6) 71.9 ± 4.2; (7) 29.8 ± 4.2; (8) 19.8 ± 4.2; (9) 68.9 ± 4.2; (10) 70.6 ± 4.2. These studies demonstrated that incubation of bovine oocytes or spermatozoa with a RGD peptide inhibits sperm-egg binding and fertilization in vitro. These findings support the notion that the role of osteopontin in bovine fertilization may involve inteaction with integrins via its RGD sequence.

## 272 OVUM RECOVERY AND BLASTOCYST DEVELOPMENT FOLLOWING INTRACYTOPLASMIC SPERM INJECTION IN CHIMPANZEES

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In the present study, we report on oocyte collection, intracytoplasmic sperm injection and early embryogenesis in chimpanzees. Eight adult female chimpanzees, 11–27 years of age, received a single s.c. injection of 3.75 mg GnRH (Leuplin, Takeda Co. Ltd., Osaka, Japan) 1 to 3 days after the beginning of menstruation. Daily i.m. injections of hMG (Humegon, Nippon Organon K.K., Tokyo, Japan) were initiated the following day. The dose of hMG was altered from 75 to 300 IU according to serum estradiol levels. When at least one follicle of 17 mm or more in diameter was observed, 10 000 IU of hCG (Pregnyl, Nippon Organon K.K.) were administered by i.m injection. Oocytes were recovered by ultrasound-guided transvaginal follicular aspiration 30.5 to 35.5 h after hCG injection. Mature oocytes were denuded of cumulus cells by treatment with 0.1% hyaluronidase, and injected with a frozen-thawed or fresh spermatozoan using a Piezo-driven micromanipulator. Zygotes were cultured in Quinn's Advantage Fertilization Medium (Cooper Surgical, Inc., Trumbull, CT, USA) with 10 serum protein substitute (SPS) at 37°C in a 5% $CO_2$ atmosphere until the pronucleus stage. The medium was replaced by Quinn's Advantage Cleavage Medium with 10 SPS from the pronuclear to 8-cell stage, and Quinn's Advantage Blastcyst Medium with 10 SPS, thereafter. Mild ovarian hyperstimulation syndrome (OHSS) occurred in one female chimpanzee with estradiol levels of 7520 $pg\,mL^{-1}$. No oocytes were collected from 2 chimpanzees in which large follicles were observed. Thirty-five mature oocytes, one immature oocyte and 6 degenerate/fragmented oocytes were retrieved from 6 chimpanzees, including the one with OHSS. Among 35 mature oocytes injected with spermatozoa, 26 oocytes (74%) produced two pronuclei; 23 zygotes (66%) cleaved to the 2-cell stage, 22 (63%) to the 4-cell stage, 14 (40%) to the 8-cell stage, and 9 (26%) to the morula stage. Seven zygotes (20%) developed to the blastocyst stage by 120 h. There were no differences in fertilization rate or early embryogenesis between frozen and fresh spermatozoa. Results indicate that techniques used for human-assisted reproduction may be applicable to the chimpanzee to help preserve this endangered species.

## 273 EFFECT OF EPIDERMAL GROWTH FACTOR (EGF) DURING OOCYTE MATURATION ON IN VITRO PRODUCTION OF BOVINE EMBRYOS

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Medium components during in vitro maturation (IVM) can significantly influence oocyte maturation and subsequent embryo development in vitro (Rose TA and Bavister BD 1992 Mol. Reprod. Dev. 31, 72–77; Harper K and Brackett B 1993 Biol. Reprod. 48, 409–416). The aim of this experiment was to evaluate the effect of EGF during IVM on further development of bovine embryos in vitro. Bovine ovaries were obtained at a slaughterhouse. Cumulus-oocyte complexes (COC) were aspirated from follicles 2–5 mm in diameter. COC were incubated for 24 h in either of 3 maturation media: T1 ($n = 72$): modified TCM-199; T2 ($n = 45$): modified TCM-199 supplemented with 10 ng mL$^{-1}$ of EGF; or T3 ($n = 46$): modified TCM-199 supplemented with 10% fetal bovine serum (FBS). After 24 h of IVM, COC were inseminated with $2 \times 10^6$ motile spermatozoa/ml. After 18 h of gamete coincubation, presumptive zygotes were denuded and placed in culture in SOF rich in glutamine (g-SOf) for 72 h, at which time, cleavage rate (%) wass assesed (embryos with >4 cells). Subsequently, cleaved embryos were incubated for an additional 72 h in c-SOF (SOF rich in citrate and glucose). Finally, embryos were cultured in modified TCM-199 for 24–48 h, at which time blastocyst formation rate (%) was evaluated. Cleavage rates were similar between T2 and T3 but significantly greater than in T1 ($P > 0.05$; see Table 1). Addition of EGF during IVM (T2; 11/45, 24.4%) did not yield more blastocysts compared to the other two treatments (6/57, 10.5% and 10/29, 34.5%, T1 and T3, respectively). Nonetheless, T3 (with serum) had a greater yield of blastocysts compared to T1 ($P > 0.01$). Results in this study show that the addition of EGF to chemically defined media results in similar cleavage rates and blastocyst yields to those obtained when using serum during IVM. Key words: in vitro maturation, EGF, cleavage, bovine, embryo.

**Table 1. Effect of EGF and serum during IVM on cleavage rate of bovine oocytes**

| Treatments | COC *n* | Cleavage rate *n* (%) |
|---|---|---|
| T1 | 72 | 18 (25.0[a]) |
| T2 | 45 | 19 (42.2[b]) |
| T3 | 46 | 21 (45.7[b]) |

[a,b]Values with different superscripts within the same column differ significantly ($P < 0.05$).

## 274 SIMILARITY OF EMBRYOS PRODUCED BY OVUM PICK-UP AND IN VITRO FERTILIZATION IN IDENTICAL TWIN CATTLE

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The present study was designed to assess the similarity of follicular development, oocyte quality, and their subsequent development on ovum pick-up (OPU)-IVF in identical twin cattle. Four pairs of identical twin Japanese black cows (A, B pairs at 5 years old and C, D pairs at 3 years old) were kept under the same feeding and environmental conditions. OPU was performed for these cows once a week for seven continuous weeks. OPU was done by using a 7.5-MHz linear transducer with needle (17 G, 530-mm length) connected to an ultrasound scanner (SSD-1200, ALOKA, Tokyo, Japan). Oocytes were evaluated by their cumulus cell morphology, cytoplasmic color, and density. To assess the development, collected COCs were cultured for 20 h in TCM-199 supplemented with 5% calf serum (CS) in a microdroplet (volume was adjusted to 5 μL/oocyte) at 38.5°C under atmosphere of 5% $CO_2$ in air. After maturation, the COCs were inseminated with frozen-thawed semen collected from the same ejaculation of a single bull. The fertilization was performed with BO solution as described by Imai *et al* (J. Vet. Med. Sci., 2002, 64(10), 887–891). The zygotes were then cultured in CR1aa supplemented with 5% CS under the same condition of maturation for nine days. Embryo development was assessed by the cleavage rate on Day 2 and the blastocyst production rate on Days 7 to 9 (insemination day = Day 0). Blastocysts were classified according to the IETS creteria. Data were analyzed by ANOVA. A total 56 sessions of OPU were performed in this study. The overall mean number of developing follicles (larger than 2 mm in diameter), collected oocytes, and produced blastocysts were $30.3 \pm 9.2$, $20.1 \pm 9.2$ and $6.3 \pm 3.8$ (mean ± SD) per session, respectively. The mean number of developing follicles on the day of OPU were significantly different between B and D pairs ($38.6 \pm 7.5$ and $21.9 \pm 6.5$, $P < 0.01$); however, no significant difference was found within each twin. In oocyte quality, C and D pairs were significantly higher grade than the A pair. The percentages of cleaved oocytes and embryos developed to the blastocyst stage ($34 \pm 16$, $27 \pm 10$, $41 \pm 17$ and $39 \pm 24$) showed no differences among 4 pairs and within each twin. However, the percentage of Grade 1 blastocyst of B pair was significantly lower ($P < 0.01$) than that of other pairs, and C pair was significantly higher ($P < 0.05$) than that of A and D pairs ($67 \pm 25$, $41 \pm 22$, $93 \pm 10$ and $71 \pm 25$; A, B, C and D pairs, respectively). There was no significant difference within twins. These results show little statistical variation between cows of the same genetic background in the production of embryos in vitro.

## 275 FOLLICULAR ATRESIA IN SMALL, NON-FSH-DEPENDENT BOVINE FOLLICLES IS ASSOCIATED WITH INCREASED DEVELOPMENTAL POTENTIAL OF OOCYTES FOLLOWING IVP

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Oocytes from small, non-FSH-dependent follicles are associated with reduced developmental competence following in vitro embryo production (IVP) compared to oocytes from larger follicles. It has been suggested that, for small follicles, oocytes derived from atretic follicles are more developmentally competent than those from healthy follicles (Blondin P and Sirard MA, 1995 Mol. Reprod. Dev. 41, 54–62). Little is known of the characteristics of small follicles that support developmentally competent oocytes. Here we examine the development to blastocyst stage of oocytes collected from histologically-assessed bovine 2–5 mm follicles. Ovaries were obtained at a local abattoir; 4 follicles were dissected from each ovary and oocytes were recovered. A section of each follicle wall was taken and fixed in 2.5% glutaraldehyde for histological assessment of the follicle and characterization of the morphology of the follicular basal lamina by electron microscopy (Irving-Rodgers HF and Rodgers RJ, 2000 J. Reprod. Fert. 118, 221–228). Oocytes recovered from follicles underwent IVP utilizing a novel single IVP system. Oocytes were matured for 24 h (10 μL per COC) in TCM199, supplemented with FSH, hCG, FCS, cysteamine and pyruvate. Mature oocytes were inseminated with $1 \times 10^6$ sperm $mL^{-1}$ for an additional 24 h using Bovine Fertilization Medium (10 μL per COC; Cook, Australia). Following insemination, putative zygotes were stripped of remaining cells and placed within individual micro-wells prepared in 1% agar in Bovine Early Cleavage Medium, Cook, Australia. The agar (350 μL) was prepared within wells of a 4-well plate and small plugs of agar were removed to form micro-wells. The agar was over-laid with 450 μL of Early Cleavage Medium and 250 μL mineral oil, and equilibrated overnight before putative zygotes were placed individually within micro-wells. Culture was performed under 7% $O_2$, 6% $CO_2$, and 87% $N_2$ at 39°C. On Day 5 following insemination, fetal calf serum (final concentration 10% v/v) was added to facilitate blastocyst development. Blastocyst formation was assessed on Day 8. A total of 211 oocytes were cultured and 69% were from healthy follicles; 67 oocytes (32%) had developed to the blastocyst stage by Day 8. Forty-three percent of oocytes recovered from atretic follicles (28/65) had developed to the blastocyst stage by Day 8, as compared to only 27% (39/146) oocytes recovered from healthy follicles, this difference was significant ($P < 0.05$, chi-square analysis). Seventy-eight percent (14/18) of oocytes from healthy follicles with additional follicular basal lamina material (Irging-Rodgers HF and Rodgers RJ, 2000 J. Reprod. Fert. 118, 221–228) failed to develop, whereas only 44% (4/9) of oocytes from healthy follicles with a normal basal lamina failed to develop ($P > 0.08$). The present study finds a direct association between the follicle morphology and oocyte maturational potential within non-FSH dependent follicles, revealing that high levels of development (>40%) can be obtained from atretic follicles. Furthermore, differences between healthy follicles may also contribute to developmental variation.

## 276 INFLUENCE OF SPERM TREATMENTS ON BLASTOCYST DEVELOPMENT IN VITRO AND CELL NUMBER IN CATTLE

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Experiments were designed to examine the effects of developmental rate and cell numbers in embryos produced by in vitro fertilization (IVF) using sperm from 2 bulls (sperm A and B purchased from commercial sale) isolated by three methods. Cumulus-oocyte-complexes collected from ovaries harvested from a local slaughter house were matured in 50 μL droplets of serum-free M199 medium supplemented with 1 μg $mL^{-1}$ estradiol-17β, 10 μg $mL^{-1}$ LH and FSH under silicone oil at 39°C in a humidified atmosphere of 5% $CO_2$ in air. After 24 h of culture, oocytes were fertilized with the sperm treated by three different methods of isolation; percoll gradient, swim-up and glass wool filtration; a final concentration of $2 \times 10^6$ cells $mL^{-1}$ was used. At 16 h after fertilization, presumptive zygotes were co-cultured in serum-free M199 with BOEC for up to 192 h post-insemination. At 48 h and 120 h post-insemination, the cultures were fed with 25 μL of serum-free M199. The embryos were compared for their rates of cleavage at 48 h post-insemination, development to the blastocyst stage, and hatching, and also the cell number at 192 h post-insemination. Differences between treatments were analyzed using one-way ANOVA after arc-sine transformation of the proportional data of cleavage, development into blastocyst stage and hatching. Comparisons of means among treatments were performed using Tukey-Kramer multiple comparisons test. The results are summarized below. The rates of cleavage in embryos produced by IVF using sperm from 2 bulls isolated by percoll, swim-up and glass wool were not significantly different. The blastocyst development and hatching rates between sperm treatment were not significant within bull sperm A and within sperm B. However, although the hatching rate in percoll treatment of bull sperm A was higher than in bull sperm B, the difference was not statistically significant. The mean cell numbers in percoll treatment of bull sperm A (176.5 ± 7.1) were significantly higher ($P < 0.001$) than the others. In bull sperm B the cell numbers in percoll treatment were higher than the other two treatments, but the differences were not statistically significant. In conclusion, these results support the concept that sperm preparation using percoll has beneficial effects on blastocyst cell number.

**Table 1. Developmental rates of in vitro embryos using sperm from 2 bulls isolated by three methods with 4 replicates**

| Sperm source | Treatment | No. of oocytes | No. cleaved at 48 h (%) | No. of blastocysts | No. hatching (%) | Cell number at 192 h (mean ± SEM) |
|---|---|---|---|---|---|---|
| Sperm A | Percoll | 230 | 202 (87.8) | 67 (29.1)[a] | 28 (12.2)[a] | 176.5 ± 7.1[a] |
| | Swim-up | 228 | 192 (84.2) | 57 (25.0)[a,b] | 23 (10.1)[a] | 140.4 ± 4.7[b] |
| | Glass wool | 233 | 194 (83.3) | 53 (22.7)[a] | 14 (6.1)[a,b] | 131.6 ± 3.6[b] |
| Sperm B | Percoll | 228 | 194 (85.1) | 40 (17.5)[a,b] | 13 (5.7)[a,b] | 143.6 ± 13.5[a,b] |
| | Swim-up | 229 | 172 (75.1) | 38 (16.6)[b] | 9 (3.9)[b] | 147.3 ± 7.0[a,b] |
| | Glass wool | 230 | 165 (71.7) | 53 (15.2)[b] | 16 (7.0)[a,b] | 133.4 ± 8.3[b] |

[a,b]Values with different superscripts within a column differ significantly ($P < 0.05$).

## 277 ENERGY REQUIREMENT DURING DEVELOPMENT TO THE BLASTOCYST STAGE OF PORCINE EMBRYOS PRODUCED IN VITRO

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A successful in vitro production (IVP) of porcine blastocysts, which enables piglet production after transfer to recipients, was reported (Kikuchi *et al.*, 2002 Biol. Reprod. 66, 1033–1041). Generally, in the IVP system, both glucose and glutamine as energy sources were included in vitro culture (IVC) medium from Day 2 (Day 0 = the day of in vitro fertilization) until Day 6. However, the exact requirement of these substances for the development to the blastocyst stage of IVP embryos has not yet been clarified. The objective of the present study was to evaluate whether these two substances are necessary for embryonic development to the blastocyst stage in culture during the period. Porcine cumulus-oocyte complexes were matured for 46 h and fertilized in vitro as reported by Kikuchi *et al.* (see above). After removal of cumulus cells and spermatozoa, the oocytes were cultured subsequently in NCSU-37 supplemented with pyruvate and lactate (IVC-PyrLac) for 2 days. Then they were cultured until Day 6 in other IVC medium prepared as follows (1–6); Basic IVC medium (BM) was a modified NCSU-37 consisting of 108.7 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25.1 mM $NaHCO_3$ and 4 mg $mL^{-1}$ fatty acid-free BSA. Then one or more of the following energy sources were supplemented to BM; (1) 12 mM sorbitol (SigmaUltra), 5.55 mM glucose (Wako special grade) and 1.0 mM glutamine (Sigma) (NCSU-37/Gln+), (2) 19.2 mM sorbitol and 1.0 mM glutamine (IVC-Sorbitol/Gln+); (3) 19.2 mM mannitol (SigmaUltra) and 1.0 mM glutamine (IVC-Mannitol/Gln+), (4) 12 mM sorbitol and 5.55 mM glucose (NCSU-37/Gln−); 5) 19.2 mM sorbitol (IVC-Sorbitol/Gln−); and 6) 19.2 mM mannitol (IVC-Mannitol/Gln−). The osmolarity of these media was adjusted to 283–285 osmol $g^{-1}$. All embryos were fixed as whole mounts, stained and evaluated. The rate of blastocysts in NCSU-37/Gln+ (26.8%) was significantly higher ($P < 0.05$; by analysis of variance and Duncan's multiple range test) than those in IVC-Sorbitol/Gln+, IVC-Mannitol/Gln+ and NCSU-37/Gln− (19.0%, 17.0% and 15.5%, respectively). A remarkable decrease in the rates in IVC-Sorbitol/Gln− and IVC-Mannitol/Gln− ($P < 0.05$; 1.4% and 2.0%, respectively) was observed. The cell numbers of NCSU-37/Gln+, IVC-Sorbitol/Gln+, IVC-Mannitol/Gln+ and NCSU-37/Gln− (55.5, 52.0, 49.6 and 58.7, respectively) had a tendency to be higher than those of IVC-Sorbitol/Gln− and IVC-Mannitol/Gln− (38.0 and 35.2, respectively). These results confirm that the supplementation of maturation medium with at least one energy source (glucose or glutamine) promotes embryonic development in vitro to the blastocyst stage, that the combination of both sources improves the chance of the embryonic survival, and that porcine embryos do not utilize sorbitol or mannitol as an energy source. The importance of glucose and glutamine is suggested for the development to the blastocyst stage of porcine IVP embryos.

## 278 EFFECT OF GLYCOSAMINOGLYCAN SUPPLEMENT ON PORCINE PREIMPLANTATION EMBRYO DEVELOPMENT AND EXPRESSION OF RECEPTORS FOR GLYCOSAMINOGLYCANS IN PORCINE EMBRYOS

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The present study investigated the effect of glycosaminoglycan (GAG) supplement on developmental competence of porcine in vitro fertilized (IVF) embryos and GAG receptor expression in the porcine embryos. In vitro-matured oocytes were inseminated with frozen-thawed boar semen and cultured in North Carolina State University (NCSU)-23 medium in the absence or presence of various GAGs (hayaluronic acid, heparin or both). Developmental competence was evaluated by monitoring the numbers of 2-cell embryos and blastocysts at Days 2 and 7, respectively. Differential staining was performed in blastocysts at Day 7. All data were analyzed by ANOVA using a Generalized Linear Model (SAS). Inseminated oocytes were cultured in NCSU-23 supplemented with different concentrations (0, 0.1, 0.5 or 1.0 mg $mL^{-1}$) of hyaluronic acid (in Experiment 1) or heparin (in Experiment 2). Supplementing NCSU-23 with 0.5 mg $mL^{-1}$ hyaluronic acid significantly increased ($P < 0.05$) the total number of cells (55.9) and the number of trophectoderm (TE) cells (41.7) compared with the other culture groups (44.7, 45.0 and 31.3, 31.8, respectively). The rate of blastocyst formation was significantly increased ($P < 0.05$) in the 1.0 mg $mL^{-1}$ heparin-supplemented group (21.8%) compared with that in the control culture group (16.4%). In experiment 3, inseminated oocytes were cultured in NCSU-23 supplemented with 0, 0.1 mg $mL^{-1}$ heparin, 0.5 mg $mL^{-1}$ hyaluronic acid, or 0.1 mg $mL^{-1}$ heparin + 0.5 mg $mL^{-1}$ hyaluronic acid. The rate of blastocyst formation was significantly increased ($P < 0.05$) in the 1.0 mg $mL^{-1}$ heparin group (21.5%) and the 1.0 mg $mL^{-1}$ heparin + 0.5 mg $mL^{-1}$ hyaluronic acid group (22.8%) compared with that of control culture group (16.6%). In experiment 4, total RNA was prepared from oocytes, 2-, 4-, and 8-cell stages, morulae, and blastocysts and reverse-transcribed using the First-strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). The cDNA was subjected to polymerase chain reaction with primers specific for hyaluronic acid receptor (CD44) and heparin binding protein (HIP). The expression of CD44 gene was detected in oocytes, and in 2- and 4-cell stages; HIP gene was detected in all preimplanatation-stage embryos. In conclusion, the present study demonstrated the embryotrophic role of GAG and expression of GAGs receptors in porcine IVF embryos. This study was supported by the Advanced Backbone IT Technology Development (IMT 2000-C1-1).

## 279 VASCULAR MORPHOMETRY OF BOVINE PLACENTOMES IN LATE GESTATION FROM EMBRYOS PRODUCED IN VIVO OR IN VITRO

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The role of the vascular supply in the development of placentas from embryos produced in vitro is poorly understood. The objective of this study was to determine the effects of in vitro embryo production on morphometry of blood vessels within fetal (cotyledonary) and maternal (caruncular)

components of the placentome during late gestation. In vivo-produced embryos were recovered from superovulated Holstein cows on Day 7 after estrus. For in vitro embryo production, oocytes were aspirated from the ovaries of Holstein cows, matured in vitro, and then fertilized. Presumptive zygotes with their cumulus cells were transferred into M-199 with 10% estrus cow serum and cultured for 168 h post-insemination. Semen from the same Holstein sire was used for the production of in vivo and in vitro embryos. Single blastocysts from each production system were transferred into the uteri of heifers. On Day 222 of gestation, fetuses and placentas were recovered in utero (in vivo, $n = 12$; in vitro, $n = 12$). Placentomes were collected, fixed and sectioned. Fetal and maternal blood vessels were identified within placentome sections using immunocytochemistry for vascular endothelial growth factor (VEGF) protein. A total of $4.8 \times 10^5$ $\mu m^2$ of tissue were examined from each placentome. Stereological methods were used to determine the volume densities of fetal and maternal blood vessels. Data were analyzed by GLM procedures. Fetuses were heavier ($P = 0.03$) in the in vitro group ($20.7 \pm 1.0$ kg, LS mean $\pm$ SEM) compared to the in vivo group ($17.3 \pm 1.0$ kg). Placentas were also heavier ($P = 0.06$) for the in vitro group ($2.5 \pm 0.2$ kg) compared to the in vivo group ($2.0 \pm 0.2$ kg). Placental efficiency, calculated as fetal weight/placental weight, was similar between the two treatment groups ($9.0 \pm 0.5$ and $8.9 \pm 0.5$ for in vivo and in vitro, respectively). Fetal vascular volume density in placentomes was not different between the two treatment groups ($5.4 \pm 0.3\%$ and $5.4 \pm 0.3\%$ for in vivo and in vitro, respectively). In contrast, maternal vascular volume density was greater ($P = 0.02$) for placentomes in the in vitro group ($5.9 \pm 0.3\%$) compared to in vivo controls ($4.9 \pm 0.3\%$). In summary, compared to placentomes from embryos produced in vivo, placentomes from embryos produced in vitro had similar volume density of fetal vessels, but had significantly increased volume density of maternal vessels. Supported by the State of North Carolina.

## 280 LAMBS BORN AFTER IN VITRO EMBRYO PRODUCTION FROM PREPUBERTAL LAMB OOCYTES AND FROZEN-THAWED UNSORTED AND SEX-SORTED SPERMATOZOA

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Developments in sperm sexing technology have resulted in the birth of a number of offspring after IVF of oocytes from adult animals (Johnson LA, 2000 An. Reprod. Sci. 60–61, 93–107). The aim of this study was to combine sperm sexing technology with juvenile breeding. Merino lambs, 2–3 weeks ($n = 43$) were hormone stimulated (Morton KM *et al.*, 2003 Proc. Soc. Reprod. Fert., P18), and COCs were matured in TCM-199 (Sigma) with 10 $\mu g\,mL^{-1}$ p-FSH (Folltropin-V; Bioniche Animal Health Australasia), 10 $\mu g\,mL^{-1}$ pLH (Bioniche), and 20% sheep serum (v/v) in a humidified 6% $CO_2$, 5% $O_2$, 89% $N_2$ atmosphere for 22 h. Semen collected from Merino rams was diluted and frozen as pellets (Unsorted), or stained with H33342, separated into X and Y sperm using a SX MoFlo (Cytomation Inc., Fort Collins, CO, USA), and frozen as pellets (Sorted). Sperm were prepared for IVF by swim-up under 0.5 mL of SOF with 2% sheep serum (v/v; SOF+) for 45 min (Unsorted), or diluted in 0.5 mL of Sydney IVF Sperm Buffer (Cook IVF, Brisbane, Australia) and centrifuged at 650*g* for 3 min (Sorted). After IVM, oocytes were transferred to SOF+, and cultured with $0.5 \times 10^6$ $mL^{-1}$ (Unsorted) or $1.0 \times 10^6$ $mL^{-1}$ (Sorted) motile sperm for 18 h. Presumptive zygotes were transferred to Sydney IVF cleavage and blastocyst medium (Cook IVF) for 3 and 5 days, respectively. Oocyte maturation and fertilization were assessed by orcein staining 18 h post-insemination (hpi). Two Day-7 blastocysts were transferred to each recipient ewe ($n = 9$; 3 per group) and pregnancies diagnosed by ultrasound on Day 57 of gestation. Data were analyzed by chi-square test. Oocyte maturation was 83.9% (73/87), and monospermic fertilization did not differ for Unsorted (22/32; 68.7%), X- (6/14; 42.8%), and Y-sperm groups (15/27; 55.6%). Polyspermic fertilization was 9.4% (3/32) and 7.4% (2/27) for the Unsorted and Y groups. Cleavage was reduced with X- and Y-sperm compared with Unsorted, but blastocyst formation (from cleaved oocytes) did not differ (Table 1). There were three (100%), zero (0%), and one (33.3%) pregnancies from Unsorted, X- and Y-embryos, respectively, all of which survived to birth, demonstrating that juvenile breeding can be successfully combined with sperm sexing.

**Table 1. Cleavage and blastocyst formation after IVF with Unsorted, X- or Y-sperm. Values in parenthesis are percentages**

| Group | No. oocytes | No. oocytes cleaving (%) | | Blastocyst formation (%) | | |
|---|---|---|---|---|---|---|
| | | 24 hpi | 48 hpi | Day 6 | Day 7 | Day 8 |
| Unsorted | 632 | 217 (34.3)[a] | 332 (52.4)[a] | 62 (18.7)[a] | 115 (34.6) | 132 (39.8) |
| X | 556 | 113 (20.3)[b] | 206 (37.1)[b] | 19 (9.2)[b] | 56 (27.2) | 68 (33.0) |
| Y | 551 | 88 (16.0)[b] | 171 (31.0)[b] | 31 (18.1)[a,b] | 68 (39.8) | 76 (44.4) |

[a,b]Values within columns with different supersripts differ significantly.

## 281 REDUCTION OF POLYSPERMY IN PORCINE IN VITRO FERTILIZATION BY MODIFIED SWIM-UP METHOD

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In vitro production (IVP) of porcine embryos facilitates research related to biotechnology and biomedicine. Even though many attempts have been made to optimize the IVP of porcine embryos, the outcome is still unsatisfactory compared to other species, such as mouse and cattle. The

high incidence of polyspermic fertilization is one of the major causes lowering the overall efficiency of porcine IVF. The common procedure for fertilization in vitro involves the co-culture of both gametes in the medium drop, which increases sperm concentration and incidence of polyspermy. Therefore, the present study was carried out to increase the efficiency of porcine IVF by reducing polyspermy using a modified swim-up method. This method modifies conventional swim-up washing by placing oocytes directly at the time of washing. Porcine oocytes were aspirated from ovaries and matured. Sperm pellet was prepared in the tube and mature oocytes were placed on a cell strainer with 70-μm pore size (Falcon 2350) at the top of the tube. After fertilization, the oocytes were fixed and stained for examination. Also, the developmental potential of fertilized embryos was measured to evaluate for the feasibility of this method. While penetration rates were similar in both methods (86.67 ± 2.36% to 83.33 ± 1.36%), there was a significant reduction of polyspermy in the modified swim-up method (17.50 ± 1.60%) compared to the control (44.1 ± 3.70%) ($P < 0.05$). Subsequent culture showed higher rate of blastocyst formation in the modified swim-up method (20.44 ± 0.99%) than in the control (15.73 ± 3.26%) ($P < 0.05$), even though the difference was not significant. These results suggest that, by controlling the number of spermatozoa reaching the oocytes, porcine oocytes might be protected from polyspermy in vitro. Also, the developmental potential of the fertilized embryos using this method could be improved by increasing the pool of spermatozoa with better quality. Further optimization of the procedure is required to impliment this method in routine porcine IVF.

## 282 MATURATION MEDIUM EXCHANGE IS EFFECTIVE ON BOVINE EMBRYO DEVELOPMENT IN VITRO

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In vitro embryo development is strongly influenced by IVM conditions. Increased duration of IVM may cause aging of the oocytes, which has a harmful effect on the embryo development. Oocyte maturation depends upon the synthesis of several proteins that may play important roles in the cytoplasmic maturation. These experiments were conducted to determine the effect of IVM duration(18-h or 24-h) and medium exchange (at 18 h) on embryo development, and to investigate the protein quantities in IVM medium. Korean Native Cow (KNC) ovaries were obtained from a local slaughterhouse, and cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm follicles. Groups of 15 COCs were matured in 50-μL drops of TCM-199 supplemented with 10% fetal calf serum (FBS), 1 $\mu g\,mL^{-1}$ MFSH, 10 μg mL LH and 1 $\mu g\,mL^{-1}$ Estradiol-17β for 18 h or 24 h. In vitro-matured oocytes were fertilized using frozen-thawed percoll separated spermatozoa (Day 0) in fer-TALP medium for 20 h and cultured in CR1aa medium supplemented with 0.3% BSA (before Day 3) or 10% FBS (After Day 3). All types of cultures were carried out in an incubator at 39°C, 5% $CO_2$ in air. The total protein quantity in IVM medium at 18 h or 24 h were compared by 2-dimensional gel electrophoresis using a 10–15% polyacrylamide gradient gels. Data from three replicates were analyzed by chi-square test. The proportions of oocytes reaching the blastocyst stage was significantly higher in 18 h IVM group than 24 h IVM group (Table 1). However, there was no difference detected in blastocyst rate between 18 h IVM group and 18 h medium exchange group. Total protein quantity was reduced between 18 h and 24 h in IVM medium. There were 299 protein spots identified in IVM medium; there was an increase at 10 spots in the IVM medium analyzed at 18 h and a decrease of 20 spots at 24 h. This study suggests that duration of IVM affects subsequent embryo development. The total protein quantity was decreased between 18 h and 24 h in IVM medium. These proteins may be absorbed into the oocytes and reduce development to the blastocyst stage. However, this may be overcome by IVM medium exchange.

**Table 1. Effects of duration of IVM and medium exchange on embryo development of KNC oocytes**

| Total maturation time | Medium exchanging | No. of examined oocytes | >2-cell | 8-cell | Blastocyst |
|---|---|---|---|---|---|
| 18 h | No | 121 | 94 (77.4 ± 0.7)[a] | 59 (48.8 ± 2.5)[a] | 31 (26.0 ± 1.1)[a] |
| 24 h | No | 118 | 80 (67.7 ± 8.6)[a,b] | 27 (22.9 ± 3.2)[b] | 16 (14.4 ± 4.6)[b] |
| 24 h | Yes (18 h) | 113 | 74 (65.6 ± 5.6)[b] | 36 (31.9 ± 12.8)[b] | 27 (23.0 ± 3.9)[a,b] |

[a,b]Values in the same column with different superscripts are significantly different ($P < 0.05$), Mean ± SEM.

## 283 ASSESSMENT OF CALVES PRODUCED BY IVP IN A SEMI-DEFINED MEDIUM

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Heavy birth weight, increased calving difficulty, heart function defects, increased perinatal mortality and organ immaturity have been reported for calves produced from IVP embryos compared to those produced from MOET or AI (van Wagtendonk AM *et al.*, 2000 Theriogenology 53, 575–597; Jacobsen H *et al.*, 2002 Anim Reprod Sci 70, 1–11). In this study we examined birth weight (BWT), and blood chemistry at 1 day of age, gestation length and heart function at 7 days, and response to an ACTH challenge at 21 days of calves derived from IVP in a 'semi-defined' IVC system (Thompson JG *et al.*, 2000 J. Reprod. Fertil. 118, 47–55) and of contemporary MOET or AI calves. Holstein Friesian (HF) 2- and 3-year-old recipients carrying single HF calves (101 × IVP and 21 × MOET) were monitored in this study. Within 1 day of birth the calves were weighed and a blood sample taken for analysis. At 7d, ultrasound measurement of the left ventricle diastolic diameter (LVEDd) and % ejection fraction (EF%) was determined. Each calf was then transported to a rearing unit. At 3 weeks of age, 30 IVP and 30 control AI calves of the same age were

injected i.v. with Synacthen (synthetic ACTH, Ciba Corporation, 0.1 $\mu g\,kg^{-1}$ body weight). Blood samples were collected at −30, 0, 30, 60 and 90 min (0 min = time of injection) for cortisol measurements. There was no difference in BWT for MOET or IVP calves (40.9 ± 4.7 v. 35.6 ± 4.8 kg, respectively). Moreover, gestation lengths (279 days v. 281 days) and calving assistance scores (1.3 v. 1.6) did not differ. Calf mortality at birth was higher for IVP calves (16%) than for MOET calves (5%). All but 7 surviving calves (6 × IVP and 1 × MOET) had high GGT levels at 1 day. Blood chemistry revealed no differences between the calf types, all measures being within normal ranges. For all calves, heart function analysis revealed no abnormalities with mean LVEDd = 4.1 ± 0.6 cm and mean EF% = 78.5 ± 8.4%. All calves exhibited elevated cortisol following ACTH challenge. There was no difference between control and IVP calves for mean cortisol concentration at any time point (0 min, 13.8 ± 5.2; 30 min, 46.6 ± 9.8; 60 min, 42.8 ± 9.9; 90 min, 28.1 ± 8.9 $ng\,mL^{-1}$). These data suggest that, unlike calves produced in less defined culture systems, calves produced by IVP in a semi-defined culture system have birth weight and gestation lengths similar to those of MOET calves. Moreover, no abnormalities in organ (heart, adrenal) function were detected. However, of concern was the high number of unexplained deaths for IVP calves. This may be due to an overall lack of vigour in IVP calves that, in an unsupervised calving, results in calf death. More vigilence at calving may be needed to ensure calf survival. The authors thank Juliet Jensen, Waikato Hospital, for ultrasound measurements and David Stewart, Morrinsville Veterinary Services, for calf care. This study was funded by Vialactia Biosciences and FRST.

## 284 THE EFFECT OF FEEDING PROPYLENE GLYCOL TO DAIRY COWS DURING THE EARLY POSTPARTUM PERIOD ON SERUM INSULIN CONCENTRATION AND THE RELATIONSHIP WITH OOCYTE DEVELOPMENTAL COMPETENCE

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High yielding dairy cows are typically in negative energy balance postpartum (pp). It has been shown that initiation of the first pp ovulation and, therefore, the resumption of normal oestrous cycles is delayed in high genetic merit dairy cows and is associated with lower circulating insulin concentration (Gutierrez *et al.*, 1999 J. Reprod. Fertil. 24, 32 abst). Evidence shows that propylene glycol (PG) rapidly elevates systemic concentrations of insulin (Bremmer *et al.*, 2000 J. Dairy Sci. 83, 2239–2251). The aim of this study was to determine the effects of PG feeding to dairy cows in the early pp period, on serum insulin and ovarian function, and on oocyte developmental competence after in vitro maturation, fertilization, and culture. Thirteen Holstein-Friesian cows were assigned to PG ($n = 6$) or control ($n = 7$) groups. Each treated cow received 500 mL of PG and each control was given 500 mL of water daily from Day 5 pp until day of AI. Blood samples for insulin were collected at 0, 30, 60 and 90 min post-drenching on Days 5, 15 and 25 pp. All cows were fed 3 kg concentrates at milking (twice daily) and had ad libitum access to a 50 : 50 maize silage : grass silage forage from the time of last blood collection. Oocytes were collected by ovum pick-up (OPU) in four sessions (following treatment with pFSH) beginning on Day 25–35 pp. The recovered oocytes were graded (Grade 1 to 4) in terms of their surrounding cumulus cells and the appearance of the cytoplasm. Grade 1–2 oocytes were matured in vitro, then fertilized using frozen-thawed bull semen, and subsequently cultured up to Day 8 in synthetic oviduct fluid. All data were analyzed using SAS version 6.12 and split-plot designs, following square root or arc sine transformation, if appropriate. PG significantly increased ($P < 0.001$) serum insulin concentration (0 min: 1.55 ± 0.19; 30 min: 4.48 ± 0.82; 60 min: 4.74 ± 0.72; 90 min: 4.10 ± 0.56) compared to the control group (0 min: 1.91 ± 0.28; 30 min 1.96 ± 0.27; 60 min: 2.37 ± 0.44; 90 min: 2.04 ± 0.26). The follicle size distribution was similar between treated and control cows for categories 2–4 mm (4.0 ± 0.47; 4.3 ± 0.70), 8–10 mm (3.2 ± 0.47; 2.5 ± 0.39), and >10 mm (0.42 ± 0.12; 0.67 ± 0.17). However, there were significantly more follicles in the 5–7 mm category (6.2 ± 0.82 v. 3.3 ± 0.43; $P < 0.05$) for treated cows. The number of follicles punctured (13.8 ± 1.02; 10.7 ± 1.04), the number of oocytes recovered (4.5 ± 0.53; 3.5 ± 0.61), and the number of Grade 1–2 oocytes (2.8 ± 0.35; 1.8 ± 0.35) were not different between treated and control cows. Although cleavage rate (68.3 v. 58.9%) and blastocyst yield (25.3 v. 14.4%) were higher for treated cows, the differences were not significant. In conclusion, these results indicate that feeding cows with PG during the early pp period increased the circulating insulin concentration. However, the developmental competence of the recovered oocytes did not differ between the groups.

## 285 BIRTH OF PIGLETS AFTER NON-SURGICAL TRANSFER OF PORCINE EMBRYOS CULTURED IN PZM-4 WITH ALTERED CONCENTRATIONS OF AMINO ACIDS

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We previously developed an in vitro production (IVP) system for porcine embryos and obtained piglets after surgical transfer of blastocysts cultured in Porcine Zygote Medium (PZM)-4. However, the developmental competence of pig IVP embryos to the blastocyst stage is still low and further improvement of IVC medium is needed. In the present study, we evaluated the effects of the addition of glutamine (Gln), hypotaurine (HT), taurine (Tau), BME-essential (EA) and MEM-nonessential (NA) amino acids solutions to PZM-4, and the replacement of polyvinyl alcohol (PVA) with BSA on embryo development to blastocysts. Moreover, the developmental competence of IVP blastocysts after nonsurgical embryo transfer (NS-ET), using a flexible catheter (FC) for deep intrauterine insemination, was investigated. Porcine COC from prepubertal gilts were matured and fertilized in vitro, using frozen-thawed ejaculated boar semen. Presumptive zygotes were cultured in PZM-4, as a basal culture medium, until Day 5 after IVF. Data from six replicates were analyzed by ANOVA. Addition of 0.25 to 4 mM Gln to PZM-4 (containing 5 mM HT) significantly increased the percentage of embryos that developed to blastocysts (15 to 31%), with addition of 2 mM Gln significantly increasing the total cell numbers in blastocysts (43 ± 17 cells) compared with no addition (3% and 20 ± 4 cells, respectively). Addition of 1.25 to 10 mM HT to HT-free PZM-4 supplemented with 2 mM Gln (named PZM-5) significantly increased the percentage of embryos that developed to blastocysts (22 to 28%) compared with control (no HT; 4%). In the culture with HT-free PZM-5, addition of 5 mM Tau significantly increased blastocyst yield (17%) compared with

control (4%). However, Tau addition in the presence of 5 mM HT had no effect on development to the blastocyst stage. In combinations of EA and NA added to PZM-5, a single dose of EA significantly increased the percentage of embryos that developed to blastocysts (27%) compared with no dose (19%) or with a double dose of EA (20%), while a double dose of NA significantly increased the total cell numbers in blastocysts (43 ± 16 cells) compared with no NA (37 ± 6 cells). Replacement of PVA with BSA in PZM-5 had no effect on embryo development to the blastocyst stage. Crossbred sows were used as recipients for NS-ET, and had their estrous cycle synchronized by a described previously method (Yoshioka *et al.*, 2002 Biol. Reprod. 66, 112–119). Five days after hCG injection, a FC was introduced via the cervix into the uterine horn of recipients without sedation. Day-5 blastocysts cultured in PZM-5 were then transferred together with 5 ml of TALP-Hepes (45 to 50 blastocysts/recipient). Of 6 recipients, one sow became pregnant and farrowed 7 piglets. Our results indicate that the addition of amino acids to PZM-4 can improve porcine embryo development to the blastocyst stage, and that blastocysts cultured in a chemically defined medium, PZM-5, can develop to full-term following NS-ET.

## 286 SPERM–OOCYTE INTERACTION DURING IN VITRO FERTILIZATION IN THE HORSE

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In vitro fertilization (IVF) has proven to be a surprisingly unsuccessful way of producing horse embryos. The aim of this study was to investigate the interaction between sperm and the cumulus oocyte complex (COC) during IVF. In experiment 1, three IVF conditions were tested: (A) COCs recovered from slaughtered mares were categorized with respect to cumulus morphology (C: compact, $n = 86$, or E: expanded, $n = 55$) and matured in TCM199 containing 0.01 IU/mL porcine FSH and equine LH (IVM); after IVM, the oocytes were denuded and those with a visible polar body were incubated with sperm (IVF) in the presence or absence of 150 ng/mL progesterone (P4) to induce the acrosome reaction (AR); (B) IVM oocytes from C-COCS were denuded ($n = 52$) or not ($n = 67$) before IVF in the presence of P4; (C) in vivo-matured oocytes ($n = 15$) recovered by transvaginal ultrasound-guided aspiration from preovulatory follicles 32 h after the donor mare was treated with hCG, were fertilized in vitro in the presence of P4. In all cases, IVF was performed with frozen-thawed, Percoll-selected sperm from a single stallion, at a final concentration of $1 \times 10^6$ spermatozoa/ml in fertil-TALP for 20 h (Parrish *et al.*, 1988 Biol. Reprod. 38, 1171–1180). In experiment 2, the possibility that semen cryopreservation or stallion critically influenced IVF was examined by incubating denuded IVM oocytes with fresh or frozen/thawed sperm from the same (fresh; $n = 17$ for both C- and E-COCs and frozen-thawed; $n = 12$ and 21 for C and E-COCs, respectively) or one other stallion (Fresh; $n = 12$ and 19 and frozen-thawed; $n = 12$ and 19 for C and E-COCs, respectively), in the presence of P4 for 20 h. In both experiments, the resulting sperm-oocyte complexes were fixed, permeabilized and labelled with fluorescein-conjugated peanut agglutinin (EY Laboratores, San Mateo, CA, USA) and ethidium homodimer (Molecular Probes, Eugene, OR, USA) to stain the acrosomal membrane and DNA, respectively, so that membrane status and position of the sperm within the oocyte investments could be detected by confocal laser scanning microscopy. The total number of sperm bound per oocyte was compared between treatments using one-way ANOVA with pair-wise multiple comparison (Bonferroni *t*-test). Despite binding to the zona pellucida (ZP), neither fresh nor frozen/thawed sperm from either stallion acrosome-reacted or penetrated any oocytes, irrespective of cumulus morphology at the onset of IVM, denudation prior to IVF or the presence of P4. However, more sperm bound to the ZP of cumulus-denuded IVM oocytes (65 ± 32 and 62 ± 28 [mean ± sd] for C and E-COCs, respectively), than cumulus-intact IVM (5 ± 4) or in vivo-matured oocytes (23 ± 17: $P < 0.001$). None of the other factors investigated affected bound sperm numbers. In all cases, ZP-bound sperm failed to AR in the classical fashion, and all oocytes remained arrested at the MII stage. In summary, fertilization failed because sperm did not acrosome-react after binding to the ZP. It is concluded that failure to adequately activate stallion sperm is an important obstacle to successful IVF in horses.

## 287 IN VITRO PRODUCTION OF HOLSTEIN EMBRYOS USING SEX SORTED SEMEN

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Our objective was to explore the synergy between sexed semen and in vitro embryo production and assess benefits of these technologies on commercial farms. Ovaries were collected from high genetic merit Holstein cull cows via colpotomy or at the time of slaughter. Oocytes were aspirated from the ovaries, fertilized 20–24 h later, and matured to the morula or blastocyst stage. Embryos were transferred into recipient Holstein cows and heifers on the same farms. Seven Wisconsin herds participated, and 365 embryos were produced from 104 donor cows. Only 272 of these embryos were transferred due to limited availability of recipients. Sexed semen from three Holstein sires was used. On average, 3.5 ± 0.37 transferable embryos were produced per donor, including 1.4 ± 0.18 grade 1 embryos and 1.5 ± 0.20 grade 2 embryos. Individual farms averaged from 1.6 to 5.8 transferable embryos per donor. Laboratory data also revealed interesting results. On average 43.7 ± 4.0 oocytes were collected per donor, and the number of usable oocytes (33.9 ± 3.4), and percent embryos cleaved (52.1 ± 1.9), were significant predicators of the number of blastocysts developed. We divided the usable oocytes and embryos cleaved per donor into quartiles. The fourth quartile for embryos cleaved was significantly greater ($P < 0.05$) than the lower three quartiles, and the usable oocyte quartiles all significantly differed from each other. Semen freeze date was also a significant predicator of the number of blastocysts developed, suggesting significant variation in the quality of sorted semen per ejaculate. To preliminarily test the effect of sorting on the percentage of embryos developing to blastocyst stage, oocytes were recovered from ovaries collected at a slaughterhouse and fertilized using non-sorted semen or sex-sorted semen from the same sires. Oocytes ($n = 3312$) fertilized using non-sorted semen tended ($P = 0.06$) to produce more embryos developing to blastocyst stage than oocytes ($n = 1577$) fertilized using sex-sorted semen (20.1 ± 2.9% v. 12.2 ± 2.3%, respectively). Preliminary pregnancy results show strong farm and sire effects. Overall conception rate was 36% for heifer recipients and 18milking cow recipients. These results suggest that low cost in vitro embryo production may have promise as an early system for utilizing sexed semen in dairy cattle breeding programs.

## 288 EFFECTS OF OVIDUCTAL EPITHELIAL AND CUMULUS CELLS ON FERTILIZATION AND EMBRYO DEVELOPMENT OF PIG OOCYTES IN VITRO

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This study was carried out to investigate the effect of adding porcine oviductal epithelial cells (POEC) and also the presence of cumulus cells during in vitro fertilization on fertilization rate and subsequent embryo development of pig oocytes matured in vitro. Cumulus-oocyte complexes (COCs) aspirated from 2- to 6-mm follicles were matured in TCM 199 supplemented with cysteine, EGF, eCG, hCG, and PVA for 20–22 h, and cultured in the same medium without hormone for an additional 20–22 h. Oviducts attached to ovaries without CL were used to obtain epithelial cells. After removal of the fimbria and utero-tubal junction, oviducts were flushed with MEM supplemented with 10% FBS, and POEC clumps were cultured in the same medium for 48 h. After the completion of maturation, COCs were randomly divided into four groups; cumulus-denuded (D), cumulus-denuded with POEC (DP), cumulus-enclosed (E), and cumulus-enclosed with POEC (EP). Eight to 10 POEC clumps were co-cultured with sperm and oocytes in a 100-μL fertilization drop. Oocytes were fertilized with frozen-thawed spermatozoa for 6 h in modified Tris-buffered medium containing caffeine and BSA. Presumptive zygotes were cultured in NCSU23. Oocytes were fixed and stained for the evaluation of penetration at 12 h after IVF ($n = 549$ oocytes), and cleavage rate and blastocyst formation were evaluated at 48 and 144 h after IVF ($n = 1531$ oocytes), respectively. Results were analyzed by Duncan's multiple range test using GLM procedure in SAS. Although the sperm penetration rate in group E was lowest among all groups ($P < 0.05$), the monospermic fertilization rate was not significantly different among treatment groups (68.6–81.9%). Although the cleavage rate and percentage blastocyst in group E were significantly lower than other groups (38.1 v. 53.6, 52.0 and 44.6%, and 15.0 v. 21.2, 23.4 and 18.5% in group D, DP, and EP, respectively), blastocyst cell number was not significantly different among treatment groups (24.9–27.3) These results suggested that the presence of cumulus cells alone during fertilization interferes with sperm penetration, cleavage, and blastocyst formation and that POEC may improve both cleavage and blastocyst formation rate.

## 289 EFFECT OF FERTILIZATION TIME OF PIG OOCYTES MATURED IN-VITRO BY BOAR SPERM STORED AT 4°C

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The use of boar sperm stored at 4°C may be a useful tool for in vitro production of pig embryos. Therefore, this study was undertaken to investigate the effect of fertilization time of pig oocytes matured in-vitro by boar sperm. The sperm-rich fraction (30–60 mL) was slowly cooled to room temperature (20–23°C) by 2 h after collection. Semen was transferred into 15-mL tubes, centrifuged at room temperature for 10 min at 800*g*, and the supernatant solution was poured off. The concentrated sperm was resuspended with 5 mL of the LEN (11.0 g lactose hydrate, 20 mL egg yolk, 0.05 g N-acetyl-D-glucosamine and 100 mL distilled water) diluent to provide $1.0 \times 10^9$ sperm $mL^{-1}$ at room temperature. The resuspended semen was cooled in a refrigerator to 4°C. The medium used for oocyte maturation was TCM-199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 μg $mL^{-1}$ insulin, 2 μg $mL^{-1}$ vitamin $B_{12}$, 25 mM HEPES, 10 μg $mL^{-1}$ bovine apotransferrin, 150 μM cysteamine, 10 IU $mL^{-1}$ PMSG, 10 IU $mL^{-1}$ hCG, 10 ng $mL^{-1}$ EGF, 0.4% BSA, 75 μg $mL^{-1}$ sodium penicillin G, 50 μg $mL^{-1}$ streptomycin sulfate and 10% pFF. After about 22 h of maturation, oocytes were cultured without cysteamine and hormones for 22 h at 38.5°C, 5% $CO_2$ in air. Oocytes were inseminated with boar sperm stored at 4°C for 2 days after collection. Oocytes were coincubated for 1, 3, 6 and 9 h in 500 μL TBM fertilization media with $1 \times 10^6$ $mL^{-1}$ sperm concentration. Thereafter, oocytes were transferred into 500 μL NCSU-23 culture medium containing 0.4% BSA for further culture of 6, 48 and 144 h, fixed and stained for the evaluation of fertilization parameters and developmental ability. Data were analysed by ANOVA and Duncan's multiple range test using the SAS program. The rates of sperm penetration and male pronuclear formation were higher in the fertilization times of 6 and 9 h than in those of 1 and 3 h. The percentage of polyspermic oocytes was highest in fertilization time of 9 h compared with other incubation times. The rates of cleaved oocytes were higher in the fertilization times of 6 and 9 h (85.0 and 84.6%) compared with those of 1 and 3 h (61.1 and 76.8%). The percentage of blastocyst formation from the cleaved oocytes was highest in the fertilization time of 6 h (33.6%) than in that of 1, 3 and 9 h (11.4, 23.0 and 29.6%). Mean cell numbers per blastocyst were $32.9 \pm 3.3$, $27.6 \pm 2.7$, $26.3 \pm 2.2$ and $24.4 \pm 1.8$ in the fertilization times of 6, 9, 3 and 1 h, respectively. In conclusion, we found out that boar sperm stored at 4°C could be used for in vitro fertilization of pig oocytes matured in vitro. Also, we recommend the coincubation time of 6 h in 500 μL TBM fertilization medium with $1 \times 10^6$ $mL^{-1}$ sperm concentration for in vitro fertilization of pig oocytes matured in vitro.

## 290 EFFECTS OF HYALURONAN ON IN VITRO DEVELOPMENT OF PORCINE EMBRYOS CULTURED IN A CHEMICALLY DEFINED MEDIUM

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Hyaluronan (HA), a glycosaminoglycan present in follicular and oviductal fluids, has been related to sperm capacitation, fertilization and embryo development. We have previously developed an in vitro-production (IVP) system of porcine embryos, where porcine blastocysts can be produced by IVF and IVC in chemically defined media and can develop to full-term by transfer to recipients. The application of a chemically defined medium to IVP in pigs allows the analysis of the physical action of substances on the development of pre-implantation embryos. In the present study, the effects

of HA on the development of porcine embryos in a chemically defined medium were investigated. Porcine presumptive zygotes were produced by IVM and IVF of COC from pre-pubertal gilts and frozen-thawed ejaculated boar semen. The zygotes were cultured in Porcine Zygote Medium (PZM)-5 containing different concentrations of HA (0 [control], 1, 2, 5, 10, 20 and 50 $\mu g\,mL^{-1}$) until 6 days after IVF, and representative specimens were fixed for cell counting and transmission electron microscopy. Data of percentages and cell numbers were statistically analyzed by one-way ANOVA and Fisher's PLSD test. The percentage of embryos that developed to the blastocyst stage (15.8% [23/144] to 19.5% [27/139]) did not differ among treatments. However, addition of 5 or $10\,\mu g\,mL^{-1}$ HA increased ($P < 0.05$) the total number of cells in blastocysts (56.1 and 58.3 cells [$n = 22$ and 23], respectively) compared to control (no HA, 42.0 cells [$n = 23$]). To evaluate proliferation rates of inner cell mass (ICM) and trophectoderm (TE), embryos were cultured in PZM-5 for various periods of exposure to $10\,\mu g\,mL^{-1}$ HA. The numbers of ICM and TE cells in Day-6 blastocysts cultured in the presence of exogenous HA from Day 0 to Day 3 (18.3 and 34.4 cells, respectively [$n = 38$]) or Day 6 (17.9 and 35.9 cells, respectively [$n = 36$]) were significantly ($P < 0.05$) higher than those cultured without HA through the culture period (13.5 and 24.2 cells, respectively [$n = 26$]). In the presence of HA from Day 3 to 6, only the number of TE cells (37.1 cells [$n = 33$]) increased ($P < 0.05$), compared to PZM-5 alone. Differences in ultrastructure were noticed among blastocysts cultured with or without $10\,mg\,mL^{-1}$ HA. Blastocysts cultured with HA had mainly mature mitochondria while many mitochondria appeared morphologically immature in the blastocysts cultured without HA. Lipid droplets in the blastocysts cultured with HA seemed to be more homogeneous in comparison with those in the blastocysts cultured in PZM-5 alone. Further differences were seen in the numbers of lysosome-like structures, which were greater in blastocysts cultured with HA. This study demonstrates that exogenous HA improves cell proliferation and normality of ICM and TE in porcine embryos cultured in a chemically defined medium, depending on the exposure periods to HA. (Supported by MAFF, Japan and STINT, Sweden.)

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Minireview

# Sex-Sorted Sperm and Fertility: An Alternative View

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## ABSTRACT

**Although contemporary methods of physically separating X from Y chromosome-bearing spermatozoa are now very efficient, overall fertility rates following the use of sex-sorted sperm are not as impressive, in spite of many attempts to improve them. At the same time, there are suggestions from evolutionary biology, and from sex allocation theory in particular, that there may need to be a modification to the chance theory of sex determination in mammals. This is because it now appears that the mammalian female could have some influence on the sex of her offspring, and furthermore, that this influence could be preconceptual. If so, this could go some way towards accounting for the putative inefficiencies in fertilization following insemination with sex-sorted sperm.**

*assisted reproductive technology, fertilization, gamete biology, sperm*

## INTRODUCTION

Beginning over 100 yr ago, with the discovery of the role of the chromosomes [1], and continuing to the present day, the allocation of sex in mammalian offspring has been considered to be a matter of chance, depending on whether an X or a Y chromosome-bearing spermatozoon (X or a Y sperm) reaches the ovum first.

At first assuming, and later showing, that X and Y spermatozoa are produced in equal numbers [2], early work on sex preselection focused on finding a measurable difference between them, especially one that would lead to a reliable way of sorting them into discrete groups. If this were possible, ensuring the conception of an offspring of a particular sex could be achieved by exposing the ovum specifically to Y sperm only (for a male) and not to X sperm, or vice versa. This thinking resulted in the emergence of the sperm separation industry.

## ACHIEVEMENTS OF THE SPERM SEPARATION INDUSTRY

Early methods of sorting X and Y sperm were sometimes based on unwarranted assumptions about differences between them [3], resulting in less than adequate concentrations of the desired spermatozoa in sperm samples [4]. Contemporary separation processes are based on a sex difference in the amount of DNA in the X and Y chromosomes: a human X chromosome containing approximately 2.8% more total DNA than a Y chromosome [5]—the difference in other mammals being on the order of 3%–5%.

This difference made it possible to devise ways of more accurately separating sperm into X-enriched and Y-enriched samples than had previously been possible. Successful methods of separation, for use primarily in the dairy industry, involve flow cytometry/cell sorting after staining sperm with a fluorescent DNA-binding dye [6]. Now sometimes referred to as the Beltsville method [7], it has recently been even further refined by the addition of an orienting nozzle, which permits better alignment of the sperm during the sorting process, leading to further increases in efficiency and accuracy.

In general, contemporary sperm-sorting procedures result in skewed offspring sex ratios of between 85% and 95% accuracy [7]. Thus, in a number of different animal species, including sheep [8], dairy cattle [9], horses [10] and pigs [11, 12], once a pregnancy is established, the incidence of the unwanted sex is low.

## THE FERTILITY PROBLEM

In spite of the remarkable technical expertise that has resulted in these achievements, the success rate, in terms of total numbers of live births, is not as impressive. Key researchers acknowledge that fertility of sorted spermatozoa is "somewhat lower" [13] than that of controls, but point out that results of studies showing this difference "are confounded by using fewer sexed spermatozoa per insemination dose than normal procedures would dictate" [13].

One of the major limitations of flow cytometric sperm sorting is the sorting rate, which means that animals fertilized with sex-sorted sperm tend to receive much lower doses of sperm than animals undergoing routine artificial insemination (AI). For example, in the horse, optimized flow-sorting allows approximately $10–20 \times 10^6$ sperm to be sorted per hour; thus, it is impractical to obtain the $500 \times 10^8$ sperm that would typically be used to inseminate mares with unsorted sperm [14]. However, when similar numbers of sorted and nonsorted spermatozoa were used in an insemination trial in cows, pregnancy rates with sex-sorted spermatozoa continued to be 20%–40% lower than control, unsorted spermatozoa [15].

There also appear to be differences between species in the fertility rates using sex-sorted sperm, with some species (e.g., sheep [8], horses [10], and pigs [12]) having low pregnancy

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rates following insemination with sex-sorted sperm compared to insemination with unsorted sperm. However, such interspecies differences may reflect suboptimal insemination procedures, as well as problems with the fertilizing potential of sex-sorted sperm.

Although it can be difficult to unearth figures that give the complete picture, reports from the dairy industry indicate a somewhat higher level of achievement. Figures are usually given in terms of percentage success rate of sorted sperm compared with that of unsexed samples in routine AI. The pregnancy rate following AI varies, but can be between 63% and 72% [15]. Thus, if the pregnancy rate for AI, using unsexed sperm, was 63%–72%, the predicted pregnancy rate for sex-sorted sperm would be 38%–58%. According to a recent review, fertility rates for sex-sorted sperm are now usually in the range of 70%–80% of unsexed sperm at normal doses in well-managed herds (although, the author adds, it has been "lower in poorly managed herds") [16]. So even the predicted [16] average pregnancy rate for sex-sorted sperm in the dairy industry (70%–80% of 63%–72%) is about 51%, and can be considerably lower [9].

Some studies do give sufficient detail to make the situation clear. Following a field study in Switzerland, researchers reported fully on the results of inseminating heifers or cows with low-dose sex-sorted or nonsorted sperm [9]. For the combined group, results showed 85.3% female offspring for the sex-sorted sperm compared with 58.6% females for the nonsorted sperm, a result which, as in many earlier studies, confirmed the efficacy of the sperm-sorting procedure. However, closer inspection of the data reveals an interesting phenomenon. A total of 27 heifers received sex-sorted sperm and 27 heifers received nonsorted sperm, both at the low dose, and in similar conditions; but heifers that had been fertilized by nonsorted sperm had nearly twice as many calves as those inseminated by sorted sperm (15 and 8, respectively). In the second part of the study, cows were treated in the same way, but in this case, calving rates did not differ, with both groups having low fertility—22.1% pregnancy rate (23/104) in the group receiving sex-sorted sperm, and 23.4% pregnancy rate (16/63) in the group receiving nonsorted sperm [9].

At the present time, it is widely acknowledged that subfertility is a problem throughout the dairy industry [17], and this too should be kept in mind when considering fertility rates following sperm sorting.

## ATTEMPTS TO IMPROVE FERTILITY RATES

Many of the industry's most recent initiatives appear to be targeted at improving fertilization rates. All aspects of the procedures are being scrutinized and analyzed, including possible effects of the sorting process itself [7, 18], effects of transporting and storage of sperm [19], optimal concentration of sperm [20], possible effects of polyspermy [21], mode and timing of delivery of the spermatozoa [22, 23], and insemination site [10], but, so far, lower fertilization rates remain unexplained.

Lower pregnancy rates appear to be associated with the use of sex-sorted sperm at virtually every step in the process (i.e., low fertilization rates tend to be followed, in addition, by lower cleavage rates and lower rates of development to blastocyst stage [20]). Whatever the cause or causes, the conclusion is similar across a number of studies; namely, that "sex-sorting sperm negatively impacts the embryo's ability to develop normally" [23]. Thus, it could be argued that the procedures associated with the use of sex-sorted sperm are not yet fully refined, that results continue to improve [20], and that, along with generalized subfertility, this is a sufficient explanation for the low fertilization rates. Whatever the final outcome, there is no doubt that these are currently reasonable positions to hold.

On the other hand, if the basic model of sex determination is inaccurate, there may be a way of interpreting the data that shows fertility following the use of sex-sorted sperm to be much more successful than currently viewed. The disadvantage of this new interpretation would be that contemporary and emerging technologies might not contribute as much to efficiency gains as expected.

## DIFFICULTIES INTERPRETING THE DATA

There are several reasons why overall fertility rates could be even lower than appears from an appraisal of the data. The first is the convention of reporting the number of pregnancies rather than the number of live births. Although times differ between species, there can be wide variation in attrition rates during pregnancy [10]. Some researchers specifically mention the possibility that pregnancy losses in cattle between 1 and 2 mo of gestation may be on the order of 1%–2% higher than those observed with normal insemination doses of unsexed spermatozoa [13, 22].

Secondly, while fertility rates following sperm separation continue to be given as a percentage of those following AI with unsorted spermatozoa, it can be difficult to estimate where the most important problems lie.

Thirdly, it could be argued that low fertility rates are underreported. Negative results are less likely to be published, and researchers less likely to report them, especially if it is then suggested that they are not managing their herds properly. The fact is, as key researchers have reported, fertility is "somewhat lower. . .even under ideal conditions such as highly fertile bulls, skilled AI technicians and well-managed heifers" [6].

Finally, low fertility rates may be less apparent in the literature because of the implicit assumptions in the theoretical model of sex determination. Methodologically speaking, if the accepted model of sex determination is that it is a matter of chance, then the number of females that fail to become pregnant is perhaps only of peripheral, academic interest (although of considerable commercial interest). On the other hand, with a model of sex determination that included a role for the mother, such information would be central.

## THE STUDY OF OFFSPRING SEX RATIOS IN EVOLUTIONARY BIOLOGY

At the same time as research into sperm separation was being undertaken in andrology and reproductive physiology, evolutionary biologists were documenting atypical offspring sex ratios, both in the wild and in captivity, searching for a solution to the problem of sex allocation in mammals. In part, this work was an exploration of Fisher's frequency-dependent sex selection theory [24], and its more specific successor, the Trivers and Willard hypothesis [25], which described how an adaptive advantage could accrue to a mother in good condition if she conceived and raised a male offspring.

In the last 30 yr, theorists and field workers alike have explored the mathematics of evolutionarily stable strategies [26, 27] and searched for evidence of adaptive control of the sex ratio in mammals [28–30]. In theory, especially since many nonmammalian species appear to have precise adaptive control of offspring sex ratio [31–33], mammals should have adaptive control too, not least for the advantage that such control would bring.

Furthermore, because the mammalian mother commits more energy to the growth and development of the offspring

compared with the father, both pre- and postnatally, it would make sense for her to have some say in which sex offspring would be more likely to achieve the greater fitness benefit at any particular time.

Thus, over the years of searching, there has been an increasing emphasis on the possibility of a role for the mammalian mother. Trivers and Willard's "good condition" was later found to be related to maternal dominance, a behavioural characteristic associated with good condition [34], and shown to be underpinned by testosterone in females [35, 36]. Both good condition and maternal dominance have been shown to be associated with the conception of statistically significantly more male offspring [37, 38].

More than 1 000 research papers have now cited the Trivers and Willard hypothesis [37], and, although there are still problems [28, 39, 40], almost half the studies (48%) investigating the hypothesis found statistically significant alterations in secondary sex ratios in the predicted direction [37], and there have been various suggestions for refinements to the hypothesis that could help explain many, if not most, of the failures to confirm it [41, 42].

Out of concern for the apparent clash with the chance model of sex determination, some evolutionary biologists have argued that significantly different offspring sex ratios could be accounted for by maternally induced pre- and perinatal mortality by sex of offspring [43–46]. These include differential loss by sex during pregnancy, as well as sex-biased neglect or infanticide at birth. There is no doubt that these occur, but little evidence to support the suggestion that this could be a sufficient explanation for atypical sex ratios. On the contrary, there is now growing evidence to suggest that both avian and mammalian mothers could have a role in predetermining the sex of their offspring at, or before, conception. For example, researchers studying atypical sex ratios in the Seychelles warbler were led to surmise, counter-intuitively, that "pre-ovulation mechanisms may be the sole means of adjustment in this species" [31]. In a recent review of sex ratios in ungulates, data were found to be consistent with the predictions of the Trivers and Willard hypothesis, provided that the researchers had differentiated between pre- and postconception measures, with preconception measures being the accurate predictors [38]. Evolutionary biologists who have included hormonal measurements along with their behavioural data have also found evidence for preconceptual influences on offspring sex ratios from maternal hormones [47, 48].

In all this, maternal testosterone may yet prove to be central, because it has the potential to provide a link between the behaviours associated with dominance [35, 36, 49] and the physiological processes involved in conception [50, 51].

## A POSSIBLE MATERNAL ROLE IN SEX PREDETERMINATION

Because atypical sex ratio data has been associated almost entirely with maternal characteristics (and not with paternal characteristics, which remain largely unknown), mid-20th century researchers suggested that the uterine environment might facilitate preferential access of either X or Y sperm [52]; however, it appears that there is no evidence to support this idea [4], although some form of maternal ratification of embryonic sex may yet be found to be relevant.

A more recent, alternative hypothesis is that an ovum may be selected and developed each oestrus or menstrual cycle, already adapted to receive an X or a Y sperm [53]. This could be the most parsimonious and least costly mechanism suggested so far. One hypothesized pathway by which this could occur is currently being investigated. It involves the follicular fluid, known to have concentrations of testosterone 10 000–30 000 times higher than serum [50]. Individual samples of follicular fluid may be either high or low in testosterone, and it is these differences, it is hypothesized, that could lead to differences in the development of the zona pellucida (ZP), such that it is rendered more or less easily penetrable by, or receptive to, either an X or a Y sperm. The molecular basis for such testosterone-induced selectivity might be very subtle. For example, it is known that carbohydrates on the ZP proteins form the oocyte receptor for sperm binding, and testosterone is known to influence the glycosylation of proteins [54]. Thus, it is possible that testosterone could also subtly alter the glycosylation on ZP3 and/or ZP2 such that one or both of these molecules preferentially accepted a Y sperm. This would mean that X and Y sperm might have demonstrable differences in their oocyte receptors. However, despite there being many candidate ligands for ZP3 on sperm, the primary ligand is currently unknown; thus, it is very difficult to confirm this hypothesis experimentally.

Overall, however, the idea that mammalian mothers could have a role to play in the predetermination of the sex of their offspring is consistent with theoretical work [26, 55–57], field work [58], and laboratory work [53, 59]. If it transpires that follicular testosterone is, in part, responsible for differential development of the ZP each oestrus or menstrual cycle, this could provide a pathway for the mother to adjust the sex of the offspring to suit current conditions. This is because testosterone levels (in both males and females) are sensitive to chronic environmental stressors: male testosterone falling, but female testosterone rising as part of a female mammal's intrinsic response to stress [49]. It could be that normally distributed female testosterone, known to fluctuate over time in response to environmental stressors, might also provide a clue to solving the problem of adaptive control of the sex ratio in mammals.

## IMPLICATIONS FOR SEX SELECTION BY MEANS OF SEX-SORTED SPERM

So far, research into the efficacy of sex-sorted sperm appears to be consistent with the possibility of a maternal role in sex predetermination. Many studies have been published showing that, in spite of the most careful and consistent application of the techniques, fertility appears to be compromised. From this viewpoint, studies in which success rates are reported only in terms of numbers and sexes of live births, but do not mention the numbers of mothers that failed to become pregnant at all, do not give the information required to make a proper assessment of the efficacy of the procedures.

A further question arises from the current convention of reporting identifiable pregnancies instead of live births, since another unknown factor is the point at which an embryo of a particular sex might need to be compatible with, or possibly, ratified by, the uterine environment.

At present, it is difficult to determine whether the low fertility rates are best attributable to factors associated with the sorting process (including damage to sperm), problems with insemination techniques, or low doses. Experiments using matched doses of sorted and nonsorted sperm attempt to address the issue of total numbers of sperm used, but even these experiments cannot control for the confounding effects of sperm damage (such as premature acrosome reaction [60]) that are almost certainly introduced by the sorting process. To perform a critical trial of sperm sorting, instead of comparing sorted and unsorted sperm, the fertilization rates of sorted and sorted/recombined sperm should be compared. This experi-

mental design would control for both low dosage and sperm damage. However, to our knowledge, this definitive experiment has not been carried out.

If, on the other hand, the ovum has a role in sex allocation, then no matter how technically successful the sperm sorting techniques, fertility rates will remain low. That is to say, if an ovum is produced, each cycle, already adapted to receive an X or a Y sperm, exposure to any number of the other kind of sperm will result in failure to fertilize or failure to develop. This would mean that sex-sorted sperm were useful only when a client wished to have, say, female offspring, or nothing.

With further refinements to sperm-sorting procedures, it is still possible that the fertilizing potential of sex-sorted sperm could be high. However, an alternative explanation for the low birth rates might be the one offered here, that the fault lies in assumptions about the model of fertilization, since the ovum may be playing a role in predetermining the sex of the offspring. Controversy and doubt surround this suggestion, but, finally, it is a researchable question. Are fertility rates following sperm separation compatible with the hypothesis that there is a preconceptual, maternal influence on the allocation of sex in mammals, or will they, ultimately, confirm the chance model of sex determination?

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Review

# Current status of sexing mammalian spermatozoa

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Thousands of offspring have now been produced via artificial insemination with spermatozoa sexed by flow cytometry and cell sorting. We are unaware of any other practical approach to sexing spermatozoa that maintains fertility. Accuracy of sexing usually is 85–95% in most species, but somewhat lower with human spermatozoa. Spermatozoa are sexed in series, one at a time, at routine rates of about 3000 live spermatozoa of each sex per second for most species, and nearly twice that rate under optimal conditions for some species. Owing to various constraints and statistical considerations, there appears to be an upper theoretical limit to sexing spermatozoa of about 10 000 live spermatozoa of each sex per second with current methodology. About a quarter of the spermatozoa processed are sexed; the rest are discarded in the process or lost due to logistical constraints. Spermatozoa undergo some damage during sorting, although much less in terms of viability than with routine cryopreservation; fertility is lower with sexed than control spermatozoa. Offspring from sexed spermatozoa appear to have no more abnormalities than do controls, and both groups grow and thrive similarly. Despite high costs and complex procedures, sexing spermatozoa, usually followed by cryopreservation, is being used commercially for cattle and horse production in several countries, and is used to produce girls to avoid X-chromosome-linked genetic diseases.

## Introduction

It is now possible to predetermine the sex of offspring from a number of species before fertilization with an accuracy of 85–95% (Seidel *et al.*, 1999; Welch and Johnson, 1999; Johnson, 2000). This noteworthy accomplishment, first demonstrated convincingly by Johnson *et al.* (1989), resulted from integration of advances in many fields including chromosomal karyotyping, artificial insemination, maintenance of spermatozoa fertility *in vitro*, DNA-specific staining, flow cytometry, computer science and high speed cell sorting. Advances from these different disciplines were integrated by a body of innovative scientists co-operating from several sites including Lawrence Livermore National Laboratory, the United States Department of Agriculture Beltsville Agricultural Research Center, Cambridge University, Colorado State University, Atlantic Breeders Cooperative and the company, XY, Inc. (Johnson and Seidel, 1999; Garner, 2001).

The objectives of this review are to explain: (1) why spermatozoa bearing the X- or Y-chromosome are so similar phenotypically, but what is different between them; (2) the principles and procedures used to sex spermatozoa by total DNA content via flow cytometry and cell sorting; (3) the accuracy, speed, and efficiency of current sperm sexing procedures, particularly explaining why the majority of spermatozoa used are not included in the final sorted product; (4) the extent to which spermatozoa are damaged during current sexing procedures, including effects on fertility, and normality of calves resulting from sexed spermatozoa; and (5) methods other than measuring DNA content, of sexing spermatozoa under recent or current investigation.

## Spermatozoa differ in sex chromosome size but in little else

### *Historical perspective*

The first documented microscopic identification of sex chromosomes was by Guyer (1910). This observation, along with those of others, generated the idea that mammalian sex might be controlled by these specialized chromatin structures. A variety of techniques, mostly unsuccessful, were used in an attempt to identify which sex chromosome was contained in an individual spermatozoon, with the ultimate goal of separating these gametes. The first convincingly documented difference between mammalian X- and Y-chromosome-bearing spermatozoa was the differential uptake of quinacrine stain by human sex chromosomes (Barlow and Vosa, 1970). The heterochromatin of the Y-chromosome fluoresced more brightly than other chromosomes, including the X-chromosome; however, this

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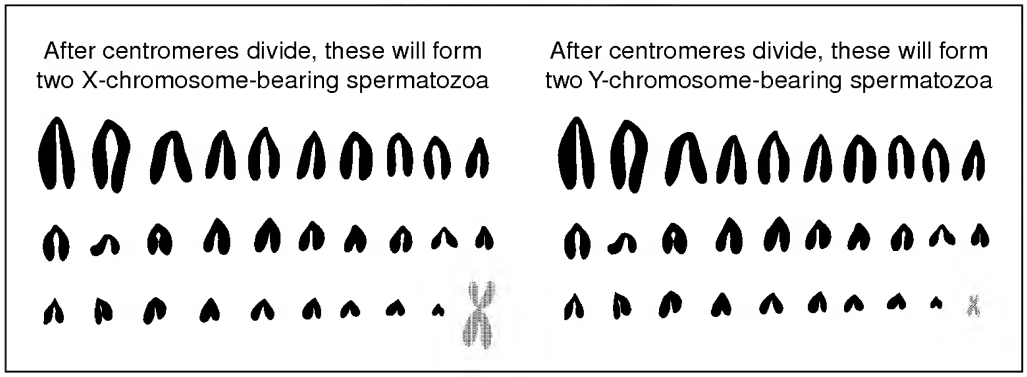


**Fig. 1.** Karyogram of chromosomes that would be present in two adjacent bovine secondary spermatocytes derived from a primary spermatocyte. The total length of chromosomes of bovine X-chromosome-bearing spermatozoa is about 3.8% greater than that of Y-chromosome-bearing spermatozoa.

difference did not occur in chromosomes and spermatozoa from non-primates.

### *50:50 Sex ratios*

Sex is determined in mammals by whether the fertilizing spermatozoon contains an X-chromosome to produce a female or a Y-chromosome to produce a male. As a consequence of the way that chromosomes segregate at meiosis, the chance that a spermatozoon will carry either chromosome is equal. Nature has gone to extremes to minimize phenotypic differences (for example, size, shape, surface properties) between spermatozoa carrying different alleles and different sex chromosomes (Seidel, 1999). Phenotypic equivalence of mammalian spermatozoa within males, despite major allelic differences, is ensured by at least four mechanisms: (1) heterochromatic sex chromosomes are encased in sex vesicles post-meiotically; (2) intercytoplasmic bridges between spermatocytes and spermatids allow interchange of molecules including mRNA; (3) there is limited post-meiotic expression of most autosomal genes during the later stages of spermiogenesis, due in part to extreme condensation of chromatin; and (4) spermatozoa are coated by high-affinity proteinaceous secretions originating from Sertoli cells, excurrent ducts and accessory sex glands, that render the surface of spermatozoa anonymous with regard to possible sex-specific and other allelic differences of the cell membrane (Seidel, 1999).

### *Karyograms*

Karyograms are displays of chromosomes at metaphase and are used to assess normalcy of numbers and shapes of chromosomes characteristic of a particular species, and to identify sex. For example, a normal male diploid bovine karyogram consists of 58 autosomes plus an X and a Y chromosome (all duplicated). A bovine karyogram is illustrated (Fig. 1). The difference in DNA content between X- and Y-chromosome-bearing bovine spermatozoa is approximately 3.8%; differences for most mammals are in the range 3–4.5% (Johnson *et al.*, 1987; Johnson, 2000) but in a few species the difference is much larger.

### *DNA-binding dyes*

The strong binding of certain fluorescent dyes to nucleic acids enables precise quantification of sperm nuclear DNA, in some cases without affecting sperm viability. Many dyes have been used, but only after application of the bisbenzimidazole Hoechst 33342 (2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2′,5′-bi-1*H*-benzimidazole·3 HCl) for staining intact spermatozoa was fluorescence quantification of the DNA content of living spermatozoa successful (Johnson *et al.*, 1987). Hoechst 33342 (H33342) is a live-cell stain that permeates the cell membrane and binds selectively to A–T base pairs along the minor groove of dsDNA. It is a yellow solid having a formula weight of 561.9 ($C_{27}H_{28}N_6O{\cdot}3HCl$) and is moderately water-soluble and relatively non-toxic. H33342 usually is excited with the 351 or 364 nm lines of an argon-ion laser or other sources of fluorescence excitation such as mercury lamps and exhibits a relatively large Stokes shift (excitation/emission maxima of about 350/460 nm), making it very useful in assessing precise amounts of DNA in cells.

Most DNA stains intercalate between the base pairs of the DNA, thereby presumably increasing their mutagenicity. However, H33342 is not an intercalative dye, which probably makes it safer to use (Watkins *et al.*, 1996). Nevertheless, H33342 may be toxic to workers at high doses, so it must be used carefully. Binding of H33342 to DNA is stabilized by a combination of hydrogen bonding, van der Waals forces, and electrostatic interactions between the dye molecule and the negatively charged DNA molecule. The strength of this multiple-binding interaction may contribute to the putative radioprotective properties of the H33342 molecule (Young and Hill, 1989).

Dead or moribund cells in the population of spermatozoa stained with H33342 can be identified by adding propidium iodide (Johnson *et al.*, 1994). More recently, this classical dead-cell stain has been replaced with red

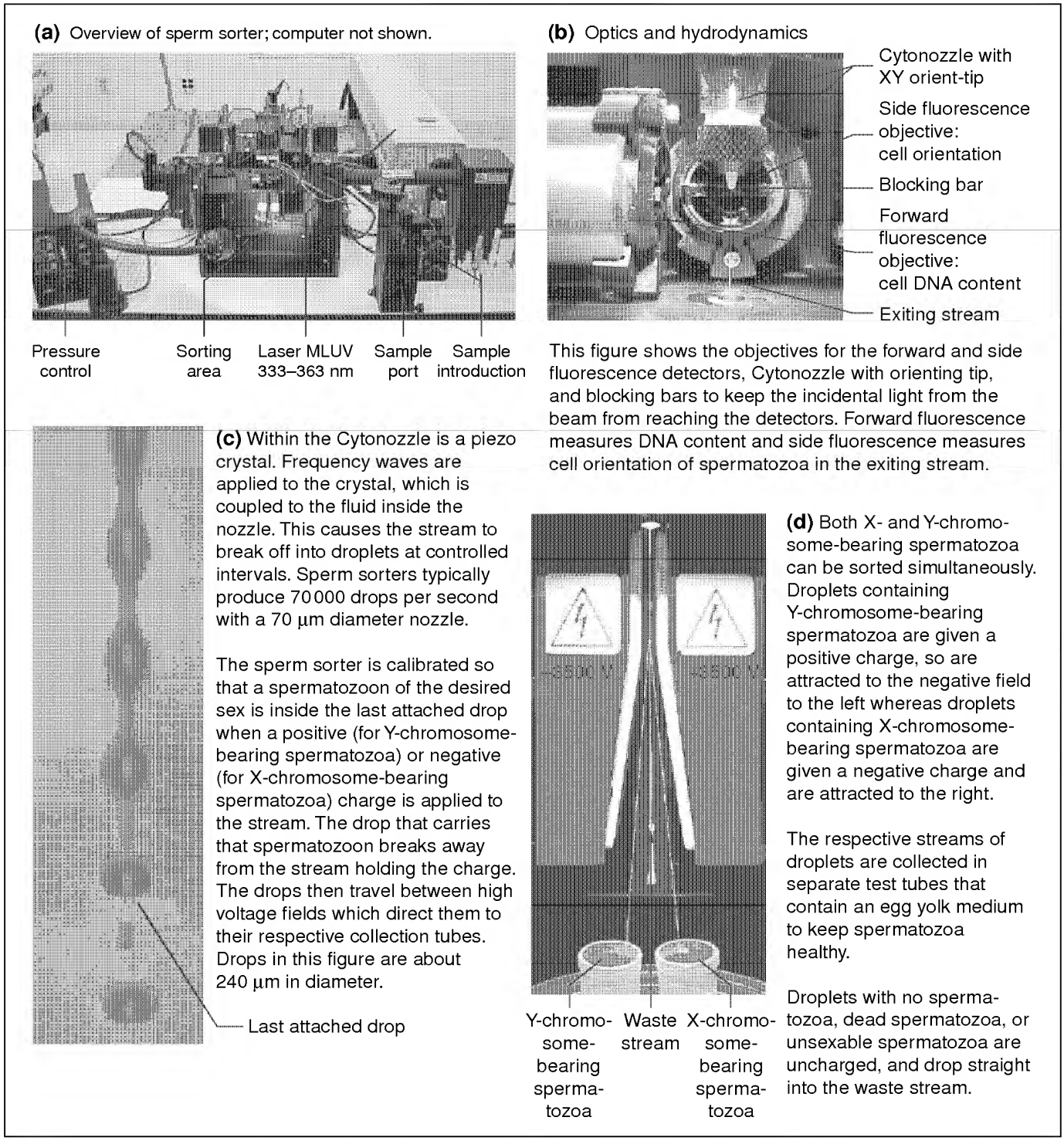


**Fig. 2.** MoFlo SX spermatozoa sorter.

food dye (FD&C40) to avoid potential mutagenic effects of propidium iodide (Johnson and Welch, 1999; Schenk *et al.*, 1999). Other food dyes also are effective. The mechanism of action is to quench the H33342 fluorescence of spermatozoa that have damaged membranes so that they can be removed during the sorting process by dead-cell gating.

## Separation of spermatozoa by DNA content via flow cytometry and cell sorting

Detailed procedures for flow-sorting mammalian spermatozoa are beyond the scope of this review, and depend on the species and application. However, an overview will be provided.

**Box 1. Steps in sexing spermatozoa**

The following steps summarize the overall process for sexing bull spermatozoa. More details are given by Schenk *et al.* (1999).

(1) Collect semen.
(2) Store up to 9 h undiluted at 20–23°C until staining.
(3) Dilute an aliquot in staining medium to $200 \times 10^6$ spermatozoa $ml^{-1}$.
(4) Stain aliquot with H33342 for 45 min at 34°C.
(5) Dilute in sorting medium to $100 \times 10^6$ spermatozoa $ml^{-1}$; add food colouring.
(6) Sort batch for 90 min.
(7) Repeat steps 3–6 every 90 min for up to 9 h from the same semen sample.
(8) Concentrate sample to approximately $40 \times 10^6$ spermatozoa $ml^{-1}$ by centrifugation at 850 ***g*** for 20 min and discard the supernatant. Sorted spermatozoa are diluted (to approximately $8 \times 10^5$ spermatozoa $ml^{-1}$) as a consequence of mixing sorting medium, catch fluid and sheath fluid (Fig. 2d).
(9) Accumulate several sort batches and pool.

### *Steps applied to semen during sorting*

The process used by XY, Inc. for sorting bull spermatozoa (see Box 1) will be used as a framework for this section. For nearly all applications, freshly collected semen is used. Spermatozoa that have been cryopreserved and thawed do not tolerate the sexing process well, particularly if they are to be re-frozen. Since sperm sorting is a slow process relative to the needs for most applications, keeping spermatozoa healthy between semen collection and sorting is important. Schenk *et al.* (1999) have done several studies on this step with bull semen, and of the many procedures tried, storing the semen undiluted at 20–23°C for 0–8 h was the best procedure. Storage of bovine spermatozoa for longer than 8 h has not been studied thoroughly in the context of flow-sorting, although limited studies with various methods have shown that dilution during storage has adverse effects on H33342 DNA-staining properties. On the other hand, stallion spermatozoa are sexed routinely after storage in appropriate diluents at 5–15°C for 18 h, resulting in excellent fertility if spermatozoa are not cryopreserved after sorting. Logistics dictate the staining of a new batch of bovine spermatozoa from the raw semen every 1–2 h, thus minimizing the time of exposure of spermatozoa to high concentrations of dye, and minimizing the destaining that occurs during dilution between staining and sorting.

In subsequent steps of the sorting process (Box 1), spermatozoa reside in a number of fluids: staining medium; sorting medium; a mixture of catch fluid, sheath fluid and sorting medium; and fluids for preparation of the final product after concentration by centrifugation, for example, cryoprotectant media. The medium for staining spermatozoa with H33342 is a modified Tyrode's albumin lactate pyruvate (TALP) (Schenk *et al.*, 1999). This is diluted with TALP containing egg yolk to which vital-staining food dye is added for sorting. During the sorting process, spermatozoa in the sorting medium are mixed with Tris-based sheath fluid containing citric acid and fructose. Sheath fluid functions as a cylindrical wall of fluid surrounding the core stream of sorting medium (containing the spermatozoa) that guides the flow of fluid through the flow cytometer. The exiting fluid is composed of approximately 90% sheath fluid and 10% core stream fluid. After spermatozoa pass through the sorter, the streams of X- and Y-chromosome-bearing spermatozoa are collected into tubes containing a 22% egg yolk-Tris extender (catch fluid; Schenk *et al.*, 1999). As the test tube fills during sorting (Fig. 2d), the initial catch fluid is continually diluted by the stream exiting the sorter nozzle. The sorting, sheath and catch fluids obviously need to be compatible with maintaining sperm fertility, but respective fluids also must have certain electrical and viscosity properties, as well as not interfere with staining and fluorescence.

Spermatozoa that accumulate every 3–4 h are frozen as a batch, so there are usually two freezing batches for each ejaculate, each freezing batch consisting of several sorting batches. In many instances, two or three sorters (up to ten at one location) are used simultaneously for the same ejaculate to improve the scale of operation. This procedure enables freezing of reasonable numbers of doses of spermatozoa per batch every 3–4 h, and minimizes the time between sorting and cryopreservation of spermatozoa.

### *Principles of flow cytometer and cell sorter use for sexing spermatozoa*

Components of a flow cytometer and cell sorter designed for sexing spermatozoa are illustrated (Fig. 2). In brief, as a spermatozoon passes two fluorescence detectors at 90° angles to each other (Fig. 2b), each detector measures the intensity of fluorescence resulting from excitation of the DNA-bound dye molecules by light, which usually is generated by a laser. The wavelengths of light used depend on the light source, and if and how the light is filtered. Importantly, if, for example, an argon laser is used as a light source, spermatozoa are not exposed to the damaging low ultraviolet wavelengths that are absorbed by nucleic acids and proteins.

The strength of the fluorescence signals obviously depends on the number of fluorescing molecules bound to DNA. This is the basis for sexing spermatozoa. In addition, the signal depends on a number of other parameters, including the laser intensity (Guthrie *et al.*, 2002), whether and how the laser is pulsed, optical properties of the entire system, sensitivity of detectors and electronic noise. All of these factors must be kept as constant as possible to resolve the small differences in DNA content between X- and Y-chromosome-bearing spermatozoa.

The main obstacle to accurate quantification of sper-

matozoa DNA with this approach is the geometry of the sperm head, which is paddle-shaped in most species of interest. The intensity of fluorescence is lowest if the flat face of the paddle is oriented toward a detector, and highest when the edge is so oriented. Flat orientation results in the most accurate discrimination between X- and Y-chromosome-bearing spermatozoa, so only spermatozoa oriented in this way are sorted (Fig. 2b). The second detector at 90° to the laser is used to diagnose orientation (Fig. 2b). As the fluorescence signal is highest for spermatozoa oriented with their paddle edge toward this 90° detector, only the population of spermatozoa that emit peak fluorescence to the 90° detector are considered oriented appropriately for sexing by the contemporaneous signal to the 0° detector (Fig. 2b).

Since sperm orientation will be random with respect to detectors in a cylindrical stream of fluid, only about 10% of spermatozoa will be sufficiently well oriented for accurate DNA measurements under those circumstances. A considerable effort, therefore, has been made to increase this percentage by modifying the cylindrical geometry of the fluid stream (Johnson and Welch, 1999; Rens *et al.*, 1998, 1999). Although details will not be reviewed here, about 70% of spermatozoa are oriented correctly with current technology (Johnson and Welch, 1999) (Fig. 2b). Other approaches to this problem, such as measuring total fluorescence with spherical detectors (Sharpe *et al.*, 1997) have not yet become useful.

### *How fast can high-speed flow cytometers and cell sorters sex spermatozoa?*

Modern flow cytometers sort spermatozoa at rates that are commercially viable by propelling spermatozoa through the system at accelerating speeds approaching 90 km $h^{-1}$ when they exit the nozzle. Discrete fluorescence signals from two detectors at 90° angles to each other are produced at a rate of over 180 000 measurements per detector per second, and the information is processed by computer and relayed to the stream-charging mechanism by the time that the spermatozoa have travelled a few cm, such that the droplets to be formed with X-chromosome-bearing spermatozoa have different electrical charges from those with Y-chromosome-bearing spermatozoa. Computation is so fast and sensitive that should a droplet contain two X- or Y-chromosome-bearing spermatozoa by chance, even these droplets can be saved, while droplets with dead or misoriented spermatozoa can be discarded.

Sorting of nearly all non-human spermatozoa for sex currently is done with the high pressure, high speed, multipurpose flow cytometer built by Cytomation, Inc. (Fort Collins, CO), model MoFlo® SX equipped with an argon laser, detectors for fluorescence, and a special spermatozoa orientation nozzle modified from one developed by Rens *et al.* (1998, 1999). Detailed technical specifications are beyond the scope of this review, but a key parameter is flow rate past the detectors, which is over 20 m $s^{-1}$. Theoretically, as spermatozoa are about 100 μm in length, if they were perfectly oriented in a column head-to-tail, about 200 000 spermatozoa could pass the detector each second, and even more if the size of sperm heads only were considered. Unfortunately, the distribution of spermatozoa in such a column approximates a Poisson distribution. This means that there is considerable space between each flowing spermatozoon. If the concentration of spermatozoa is increased, while this average space shortens, more and more spermatozoa are relatively clumped, so that for practical purposes, fluorescence signals between them are too close together for the system to process them separately, resulting in more and more unusable coincidence signals. A second, related problem is that some spermatozoa are statistically 'between droplets', so it is unclear in which droplet (Fig. 2c) the spermatozoa will reside; in this case, both drops are discarded (termed an abort). Theoretically and empirically, the optimum throughput in this statistical situation is achieved at about 25 000 spermatozoa per second, the so-called event rate. Event rates can be changed by modifying the concentration of spermatozoa in the sample, either directly or by changing the ratio of the core stream to sheath fluid. However, there is no net benefit above about 25 000 spermatozoa per second due to increasing rates of abortion or coincidence. Another option is to increase the flow rate past the detector by increasing the pressure of the entire system. However, this similarly results in increased rates of abortion or coincidence and damage to the spermatozoa.

Superimposed on the above considerations are physical constraints of droplet size. Droplets are formed as the column of fluid exits the nozzle due to vibrations set up by a piezoelectric mechanism in the column, even before the spermatozoa pass the detectors. The frequency of vibrations must be matched with characteristics of the nozzle tip, particularly diameter, as well as viscosity of the fluid and system pressure. Roughly, with a nozzle with an internal diameter of 70 μm, nearly 70 000 droplets can be formed per second at pressures of 40 psi, but fewer at lower pressures and larger orifice diameters. The ideal situation is to make as many droplets per unit time as possible without disturbing other properties of the system such as efficacy of sperm orientation.

Matching a Poisson distribution of 25 000 spermatozoa per second in the column of fluid to discrete droplets of a specific size produced at 70 000 per second occurs imperfectly. About 25% of droplets will contain a single spermatozoon, a few per cent will contain two or more spermatozoa, and > 70% will not contain any spermatozoa. There is no simple way around these inefficiencies as they are inherent due to laws of statistics and physics. They place an upper limit on sorter performance at approximately 80 000 droplets per second at 50 psi and sorting about 10 000 live spermatozoa of each sex per second per nozzle, assuming perfect sperm orientation and perfect resolution of oriented spermatozoa. A better option may be lower pressures and fewer droplets, resulting in less sperm

**Box 2. Typical efficiency of sexing spermatozoa**

Aliquot of stained spermatozoa (100%)

| | | |
|---|---|---|
| (1) | Residual loss of spermatozoa in staining tube | 10% |
| (2) | Losses in sorter tubing between batches, between males and to prevent or correct plugged nozzles | 12% |

Spermatozoa that are evaluated (78%)

| | | |
|---|---|---|
| (3) | Spermatozoa discarded due to malorientation | 30% |
| (4) | Spermatozoa discarded due to coincidence | 15% |
| (5) | Discarded dead spermatozoa | 10% |

Potentially sortable spermatozoa (35%)

| | | |
|---|---|---|
| (6) | Spermatozoa discarded to maintain purity (Fig. 3) because distributions of X- and Y-chromosome-bearing spermatozoa fluorescence overlap | 12+% |
| (7) | Probable aneuploid spermatozoa discarded (Fig. 3) | 1% |
| (8) | Spermatozoa discarded due to aborts and droplets with both X- and Y-chromosome-bearing spermatozoa | 2% |

Spermatozoa that have been sorted (30%)

| | | |
|---|---|---|
| (9) | Spermatozoa lost because of spraying (missing the fluid in the bottom of the collection tube Fig. 2d) | 4% |
| (10) | Losses of spermatozoa in the supernatant after centrifugation (concentration step) | 15% |
| (11) | Loss of spermatozoa during filling and sealing straws including incomplete volume of residue in the last straw | 4% |

Sorted spermatozoa that are frozen (23%)

| | | |
|---|---|---|
| (12) | Use of spermatozoa for quality control (accuracy and motility after thawing) | 4% |

Spermatozoa available for insemination (22%; 11% of each sex at 90% accuracy)

Percentage values in the right column refer to percentages of the respective sub-headings rather than of the starting material.

damage but slower sorting speeds. To circumvent these limits would require fundamental changes in sorting procedures, changes that seem unlikely to be developed over the next few years.

### *Efficiency of sperm sorting*

There is potential for sperm losses at virtually every step of processing and sorting (see Box 2). The losses are highly dependent on the staining properties of a particular ejaculate as well as on the skill and the care taken by the technicians doing the work. Losses are multiplicative. Typical cumulative efficiency for the 12 sequential steps in Box 2 at 90% accuracy of sorting is 22% (11% of each sex). Of course, 10% of the spermatozoa were dead and were purposely discarded.

The above efficiencies vary as a result of the quality of the sample and the speed of sperm flow through the system. Sometimes ejaculates degrade during the day and the efficiencies listed become much lower, particularly at step 1, but also at steps 5 and 6. About 5% of the time, the entire day's work is discarded because the motility of spermatozoa after thawing does not meet quality control standards, as also occurs with unsexed spermatozoa. As fewer sexed spermatozoa usually are packaged per insemination dose than conventionally, these losses do not necessarily lead to fewer inseminates per volume of semen used than occur with normal semen processing with standard numbers of spermatozoa.

Under most practical circumstances, only part of an ejaculate will be sorted because the process is slow relative to the number of spermatozoa available. As spermatozoa will undergo various stresses during the sorting process, fresh samples seem to tolerate sorting significantly better than gametes that have been stored for hours. The above considerations, plus the number of sperm sorters available will determine how many spermatozoa in an ejaculate should be held for sorting, and how many will be available for non-sorted applications.

The raw data from flow cytometers can be displayed in many ways. The simplest way is to present only the fluorescence data from the 0° detector for adequately oriented, live spermatozoa (Fig. 3), that is those showing maximal fluorescence with the 90° detector (see Johnson and Welch, 1999). Misoriented and dead spermatozoa are not considered in Fig. 3; droplets containing them and spermatozoa not resolvable due to coincidence would receive no electric charge when exiting the nozzle of the sorter and, thus, would be discarded in the waste stream (Fig. 2d). Thus, the fluorescence from the 0° detector of the oriented, live subset of resolvable spermatozoa (less than half of the spermatozoa monitored) can be plotted as in Fig. 3. Note that the distributions of fluorescence of the X- and Y-chromosome-bearing spermatozoa overlap, resulting in an overall bimodal distribution. By discarding the spermatozoa in the centre of the bimodal distribution (shaded in Fig. 3), the spermatozoa to the left are primarily Y-chromosome-bearing spermatozoa and those to the right are primarily X-chromosome-bearing spermatozoa. The width of the shaded area in Fig. 3 generally is set to produce spermatozoa sexed at 90% accuracy. Spermatozoa in the extreme tails of the curve often have missing (left tail) or extra chromosomes (right tail); these aneuploid spermatozoa also are discarded by not charging droplets containing them.

## Damage to spermatozoa and normality of calves

### *Damage to spermatozoa during sorting*

Fertility of sorted spermatozoa is somewhat lower than that of controls (Seidel *et al.*, 1999; Buchanan *et al.*, 2000) as is survival of sorted spermatozoa after cryopreservation

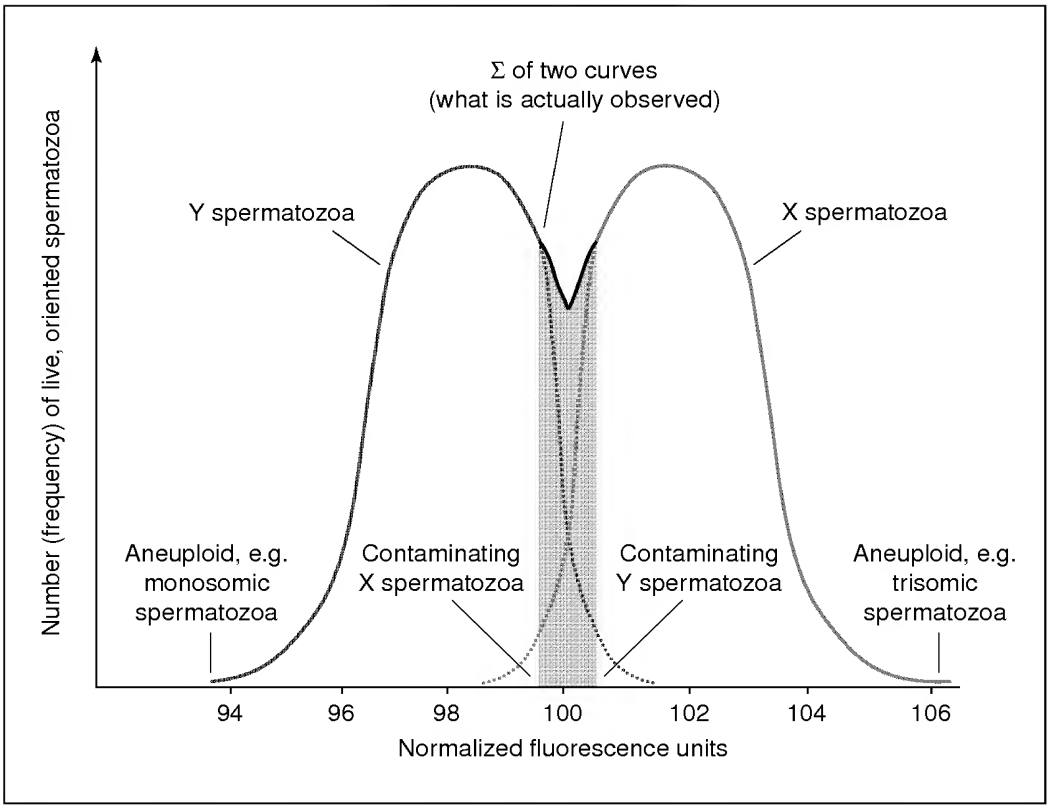


**Fig. 3.** Theoretical histograms illustrating sorting efficiencies of X- and Y-chromosome-bearing bovine spermatozoa recovered from the sorter. The initial sample would contain 50% Y- (blue) and 50% X-chromosome-bearing (pink) spermatozoa. The shaded area between the X- and Y-chromosome-bearing spermatozoa peaks show magnitude of the overlap, where it is impossible to distinguish between spermatozoa carrying an X-chromosome and those with a Y-chromosome. These spermatozoa are discarded. The wider the shaded area, the purer the X- and Y-chromosome-bearing spermatozoa populations will be, but the more spermatozoa that will be discarded.

(Schenk *et al.*, 1999). Unfortunately, most studies on fertility with sexed spermatozoa are confounded by using fewer sexed spermatozoa per insemination dose than normal procedures would dictate. When similar numbers of spermatozoa per dose have been used, pregnancy rates with sexed spermatozoa usually have been 60–80% of unsexed control spermatozoa (Doyle *et al.*, 1999; Seidel *et al.*, 1999). Pregnancy losses in cattle between 1 and 2 months of gestation have been 1–2% higher with low insemination doses of sexed spermatozoa than with normal insemination doses of unsexed spermatozoa (Seidel *et al.*, 1999). It will take large numbers of animals to determine whether this is a true effect, or whether this non-significant difference will dissipate with more thorough study.

One recent study concerns the amount of damage to DNA and mortality of spermatozoa subjected to various combinations of mechanical forces at 50 psi, exposure to laser, and staining procedures during sorting (Fig. 4; Garner *et al.*, 2001). Sorted spermatozoa were further analysed by flow cytometry for both DNA integrity (Evenson, 1989) and failure to exclude propidium iodide as a measure of dead spermatozoa. It is clear (Fig. 4) that most of the damage resulted simply from processing the spermatozoa through the sorter, even with no staining and no exposure to laser light. Additional damage due to exposure to laser and dye was small and not statistically significant, in agreement with other studies (Libbus *et al.*, 1987; Guthrie *et al.*, 2002). Very recent studies from our laboratory (Suh and Schenk, in press) indicate that much of the mechanical damage noted can be alleviated by lowering the pressure of the fluid during sorting. This is likely to improve fertility of sexed spermatozoa compared with studies to date in which spermatozoa have been sorted at 50 psi.

### *Normalcy of offspring of sex-sorted spermatozoa*

Several thousand offspring from seven mammalian species (cattle, pigs, rabbits, horses, sheep, elk and humans), primarily cattle, have been produced after H33342-staining and flow-sorting of the fertilizing spermatozoa. No gross

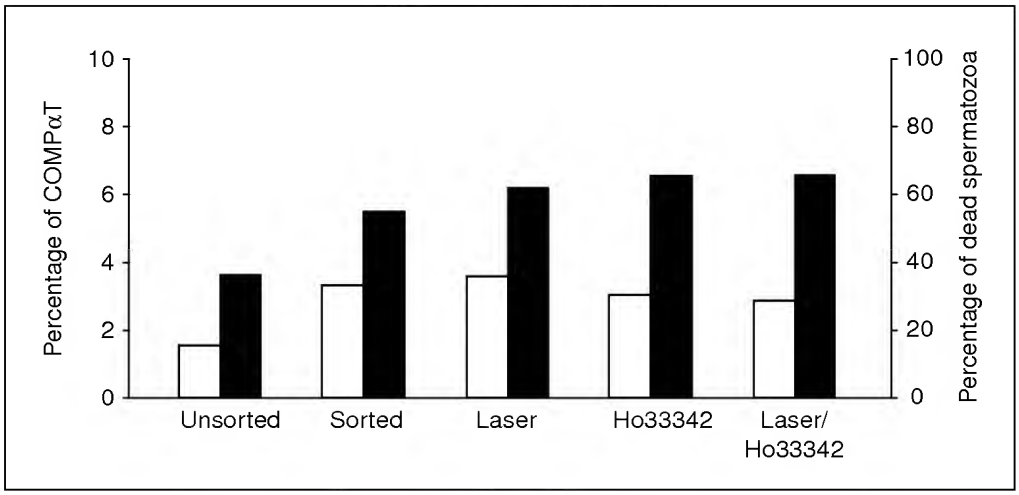


**Fig. 4.** Percentages of (■) dead spermatozoa and (□) spermatozoa with damaged DNA after thawing as determined by the spermatozoa chromatin stability assay (percentage of COMPαT, cells outside of the main population) after (1) unsorted control, (2) passing spermatozoa through the sorter without laser or staining, (3) with laser but no staining, (4) with staining, but no laser, and (5) with both staining and laser (modified from Garner *et al.*, 2001).

abnormalities have been reported (Morrell and Dresser, 1989; Johnson, 1995; Catt *et al.*, 1997; Doyle *et al.*, 1999; Fugger, 1999; Johnson and Welch, 1999; Seidel *et al.*, 1999), although we are aware of an anecdotal report of one abnormal calf. These data indicate that the DNA of flow-sorted spermatozoa that result in offspring is not severely damaged. Here, we report preliminary results from a large, ongoing study.

Black Angus heifers were inseminated with frozen, sexed and unsexed control spermatozoa during 3 days. Standard sexing procedures were used (Schenk *et al.*, 1999; Seidel *et al.*, 1999). Inseminations were balanced over semen from two bulls, four inseminators, two doses of sexed spermatozoa ($1.5 \times 10^6$ and $4.5 \times 10^6$ frozen spermatozoa per insemination dose) and a control ($20 \times 10^6$ frozen, unsexed spermatozoa), and two insemination times (12 and 24 h after oestrus). However, all inseminations were carried out 'blind' with respect to treatments. Some heifers were inseminated with X-chromosome-bearing spermatozoa, and others with Y-chromosome-bearing spermatozoa, depending on pedigrees and perceived commercial value of the resulting calves. Detailed fertility results will be reported elsewhere; briefly, there was no significant difference in 2 month pregnancy rates determined by ultrasonography between the two doses of sexed spermatozoa (overall average, 53%; $n = 245$), which, however, were lower ($P < 0.05$) than control pregnancy rates (66%; $n = 126$).

The pregnant heifers from this study were then allocated to three different farms and managed by routine husbandry procedures, but with different intensity of management. Personnel managing the cattle were unaware of which heifers were pregnant with sexed or control spermatozoa. Between 2 months of pregnancy and term, there were four abortions of 86 (4.7%) control pregnancies and seven abortions of 130 (5.4%) pregnancies from sexed spermatozoa, a very small, non-significant difference.

There were no significant differences in rates of neonatal death or accumulated deaths to weaning between calves derived from sorted versus control spermatozoa, nor were there any treatment effects on duration of gestation, birth weight or weaning weight (Table 1). No congenital abnormalities were observed. The only significant effects ($P < 0.01$) were that male calves were heavier at birth and weaning than female calves. Therefore, there appear to be no detrimental effects of sorting spermatozoa on resulting offspring. There were large differences in the survival of calves among the three farms, with deaths to weaning ranging from 5 to 18%. Such routinely observed differences in husbandry from farm to farm obviously are much greater than any effects of sexing spermatozoa. Although this data set resulting from 371 inseminations has a respectable sample size (123 calves from sorted sperm; 82 from controls), more extensive data need to be examined to be certain that there is no small increase in problems of offspring resulting from sorted spermatozoa. In addition, the fate of the minute quantity of H33342 in the female reproductive tract from the spermatozoa that do not fertilize oocytes probably merits further study.

## Methods for sexing spermatozoa other than DNA quantification with DNA binding dyes

In addition to fluorescence flow cytometry and cell sorting based on DNA content, separation of spermatozoa containing the X-chromosome from those with the Y-chromosome has been attempted with a variety of other techniques

**Table 1.** Characteristics of calves produced from frozen flow-sorted and control spermatozoa

| Treatment | Sex of calf at birth | *n* | Mean duration of gestation (days) ± SE | Number of calves that died neonatally | Mean birth weight (kg) ± SE[b] | Number of calves that died up to weaning | Mean weaning weight (kg) ± SE[b] |
|---|---|---|---|---|---|---|---|
| Sorted[a] | Male | 59 | 281.0 ± 0.72 | 2 (3.4%) | 33.5 ± 0.72 | 3 (5.1%) | 267 ± 3.4 |
| Sorted[a] | Female | 64 | 280.3 ± 0.65 | 6 (9.4%) | 30.6 ± 0.63 | 8 (12.5%) | 259 ± 3.8 |
| Control | Male | 48 | 280.1 ± 0.61 | 4 (8.3%) | 34.4 ± 0.74 | 8 (16.7%) | 267 ± 4.8 |
| Control | Female | 34 | 280.8 ± 0.70 | 3 (8.8%) | 31.2 ± 0.87 | 3 (8.8%) | 261 ± 5.9 |

[a]Accuracy of sorting was 56 of 59 males (95%) produced with Y-sorted spermatozoa, and 62 of 64 females (97%) produced with X-sorted spermatozoa.
[b]The only significant differences among treatments were that male calves were heavier than female calves ($P < 0.01$) at birth and weaning.

(Amann and Seidel, 1982). A brief update of recent and ongoing work with these technologies follows.

### *Gradient swim-down procedure*

Successful separation of X- and Y-chromosome-bearing human spermatozoa using an albumin gradient was first reported by Ericsson *et al.* (1973). The conceptual basis for this method is that Y-chromosome-bearing spermatozoa are smaller in size and exhibit a greater downward swimming velocity than X-chromosome-bearing spermatozoa within vertical columns of high density human serum albumin (Ericsson *et al.*, 1973). A fraction enriched with Y-chromosome-bearing spermatozoa can be obtained by harvesting the first 22% of spermatozoa to swim to the bottom of the gradient, and discarding the remainder (Ericsson and Ericsson, 1999). Ericsson and Ericsson (1999) reported that the latest version of this technique increased the percentage of male children born to 70–80%. However, the validity of sex pre-selection by this approach has been challenged repeatedly (for example, Evans *et al.*, 1975). This technique has never been shown to sex spermatozoa accurately from mammals other than humans (Beal *et al.*, 1984; White *et al.*, 1984). Furthermore, it is not possible ethically to do prospective, randomized, 'blind' trials with sexed human spermatozoa rigorously to document the true efficacy of this technique. Another interesting aspect of the use of this method is that when women are treated with clomiphene citrate to induce ovulation before insemination, the sex ratio is reversed, so that up to 73% females are born (Ericsson and Ericsson, 1999).

### *Surface antigenic differences*

Various immunological approaches to sexing spermatozoa of mammals have been tested without repeatable success (Hoppe and Koo, 1984; Hendriksen *et al.*, 1996; Hendriksen, 1999). One approach was to target H-Y antigen. However this molecule appears to be on both X- and Y-chromosome-bearing spermatozoa (Hoppe and Koo, 1984) and is possibly derived from Sertoli cells. An immunological approach, however, would be highly desirable because inexpensive batch processing could be used to enrich for either X- or Y-chromosome-bearing spermatozoa. Howes *et al.* (1997) suggested that their inability to detect sex-specific differences in spermatozoa surface antigenicity using rigorous biochemical methods indicates that an immunological approach to semen sexing was unlikely to work. Nonetheless, attempts using this approach recur regularly.

A recent report suggests that a viable immunological sperm sexing procedure can be developed using a more rigorous method to isolate sex-specific proteins (SSPs) (Blecher *et al.*, 1999). In this newer approach, non-SSPs were removed immunologically before the attempted isolation of SSPs because they are likely to be more highly conserved than non-SSPs. Antibodies to SSPs were raised and used to identify SSPs by affinity chromatography (Blecher *et al.*, 1999). Antibodies to purified female fetal SSPs caused agglutination of approximately half of the bovine spermatozoa and when the unagglutinated spermatozoa were isolated and used in bovine IVF, they produced > 90% male embryos (Blecher *et al.*, 1999). This immunological approach, which implies post-meiotic transcription or translation of SSPs that do not equilibrate through inter-spermatid cytoplasmic bridges, appeared promising. However, to date there are no reports of producing sex-selected offspring with this procedure despite considerable investment of resources over several years.

### *Free-flow electrophoresis*

Electrophoretic separation of mammalian sex determining spermatozoa has been attempted by many investigators without significant success (Kiddy and Hafs, 1971; Mohri *et al.*, 1987). This approach, which is based on the possibility that the electric charge on the surface of X-chromosome-bearing spermatozoa differs from that exhibited by Y-chromosome-bearing spermatozoa, uses an electric field to separate spermatozoa into the two major classes (Kaneko *et al.*, 1984). Spermatozoa are introduced into the free-flow apparatus continuously and move along different paths to be collected as two main fractions. F-body examination of separated human spermatozoa using quinacrine staining indicated that the purported X-chromosome-bearing fraction was relatively pure, but that the spermatozoa in the Y-chromosome-bearing fraction were not (Kaneko *et al.*, 1984). However, the resulting spermatozoa were compromised because sperm motility was reduced

significantly. Successful separation of sex-determining spermatozoa of other mammalian species has not been reported, although this approach may currently be undergoing reappraisal.

### *Sperm sorting based on volumetric differences*

Spermatozoa containing an X chromosome are theoretically larger than those containing a Y chromosome. van Munster *et al.* (1999a) recently used interference microscopy and subsequent image analysis to demonstrate a difference in sperm head volume that matched differences in DNA content between X- and Y-chromosome-bearing bovine spermatozoa. A method based on this principle has been developed for sorting live spermatozoa by using interference microscopy optics with a flow cytometer (van Munster, 2002). Such a method, which eliminates the need to use DNA-specific dyes, would be a highly attractive alternative method for sexing mammalian spermatozoa. Unfortunately, the potential purity of spermatozoa separated using volumetric measurements cannot exceed 80% purity of either sex based on theoretical considerations (van Munster *et al.*, 1999b), and recent efforts to make this practical have not been encouraging (van Munster, 2002).

### *Centrifugal countercurrent distribution*

Recently, Ollero *et al.* (2000) have attempted to sex ram spermatozoa by centrifugal countercurrent distribution using an aqueous two-phase system. This is a chromatographic process that partitions cells into a stationary, lower phase and a mobile, upper phase, repeated numerous times. Centrifugation was used to speed the partitioning process, so a set of 59 partitions was done in about 1 h.

Ollero *et al.* (2000) found that they could obtain fractions of up to 75% Y-chromosome-bearing spermatozoa with reasonable viability using this procedure at certain salt concentrations. However, they presented no data on repeatability of the process or fertility of the spermatozoa. Each batch produced about $6 \times 10^6$ spermatozoa (75% Y-chromosome-bearing). This procedure needs to be verified; it also may not be as successful for species with less difference in DNA content between X- and Y-chromosome-bearing spermatozoa than in sheep (4.2%; Johnson, 1995).

### *Genetic approaches*

Some years ago, a genetic approach to sperm sexing was suggested (Seidel, 1988), which subsequently has been demonstrated (Herrmann *et al.*, 1999). This approach involves the well known transmission distortion ratio of alleles at the T loci on chromosome 17 in mice. Rather than having half of the offspring with each of two alleles, > 90% (the percentage depends on the specific allele and genetic background) receive the detrimental allele, a violation of Mendel's law of independent assortment. Basically, spermatids with one allele 'poison' those with the other allele, presumably via intracellular bridges in spermatids. Herrmann *et al.* (1999) demonstrated this concept by placing part of this genetic system on the Y chromosome using transgenic procedures. They produced a strain of mice that produced 66% males (217/331) in the course of natural mating. In principle, this could be done in any species, with either sex chromosome; however, such a project would be expensive and complicated, and there are some caveats in non-murine species.

## Perspectives on commercialization

Commercialization of sperm sexing has begun for cattle and is imminent for horses (Buchanan *et al.*, 2000), and the methodology is being used on a limited scale to produce human babies, particularly to produce girls to avoid X-linked genetic disease (Johnson *et al.*, 1993; Fugger *et al.*, 1998). This review has focused on the actual process of sexing spermatozoa by flow cytometry and cell sorting, and illustrates that the procedure is complicated and somewhat inefficient. However, procedures continue to improve. Sperm sexing as currently practised is expensive, partly due to inefficiencies, partly due to personnel costs for the many steps, and partly due to the high cost of equipment and its maintenance. Despite these costs and complexities, the procedure works, and already appears to be commercially viable for niche applications in several species. Much simpler equipment designed specifically for sperm sexing probably will become available within a few years. As efficiencies improve and costs decline, sperm sexing will be applied more widely.

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# Sex-Sorting Mammalian Sperm: Concept to Application in Animals

Review

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During the last 30 years 3 major conferences have been held focusing on the prospects for regulating the sex of offspring at conception by treating or sorting sperm. The first was held in Pennsylvania (Kiddy and Hafs, 1971), the second in Colorado (Amann and Seidel, 1982), and the third in The Netherlands (Johnson and Seidel, 1999). The first conference provided insight, whereas the second two conferences reported significant scientific and technological advances in sex predetermination. We now can predetermine the sex of calves with 85%–95% accuracy by sexing sperm (Amann, 1999; Seidel et al, 1999a,b). Similar success has been reported in other species (Johnson et al, 1989; Johnson, 1991; Catt et al, 1996; Rath et al, 1996; Fugger et al, 1998; Lindsey et al, 2000). Sex predetermination came about due to advances in computer science, biophysics, cell biology, and applied reproductive physiology. This paper briefly documents how this process came about.

## Background

Many important advances occurred in the late 1970s and early 1980s. The first was accurate measurement of sperm DNA content using flow cytometry. In this measurement system, fluorochrome-stained sperm are made to flow through the system in a precise, single-file manner so that the dye that binds to each cell can be excited by a specific wavelength of light, thereby emitting a fluorescent signal. The fluorescence from each stained sperm is collected with a photomultiplier tube and the quantitative information is conveyed to a computerized system in which sperm can be categorized according to fluorescent intensity. Such a measurement system, which is capable of making precise, accurate measurements of fluorescent signals, was shown to be theoretically capable of measuring the small difference in DNA content between X- and Y-chromosome-bearing sperm (Van Dilla et al, 1977).

Early efforts to use this flow cytometric system to measure the DNA content of sperm were only marginally successful, in part because the highly condensed sperm nucleus makes quantitative fluorescence measurement difficult due to the unusual shape of the head and chromatin compaction (Van Dilla et al, 1977). This problem was conquered in the early 1980s by instruments that either oriented sperm during measurement or whose coaxial measurement principal was essentially insensitive to cell orientation (Otto et al, 1979; Pinkel et al, 1982a). A custom-made instrument used a beveled, flattened needle to hydrodynamically force each spermatozoon into a similar orientation, thereby allowing accurate measurement of the fluorescence of the flat surface of most sperm nuclei (Fulwyler, 1977; Dean et al, 1978; Figure 1). This instrumentation provided high-resolution measurements of the DNA content of mammalian sperm (Pinkel et al, 1982a). Accurate measurements of sperm DNA were essential efforts at Lawrence Livermore National Laboratory to enable sperm-based assessments to determine mutagenic damage from radiation and other environmental insults (Gledhill et al, 1976).

One early piece of evidence that flow cytometric measurements might differentiate between X and Y cells was detection of a difference in DNA content of sperm from mice with a chemically induced genetic abnormality compared with those from normal mice (Gledhill et al, 1982; Pinkel et al, 1982a). This unusual strain, called the "Cattanach mouse," had a translocation of a piece of chromosome 7 to the X chromosome (Cattanach, 1961; Disteche et al, 1981). The difference in DNA content between the bimodal peaks of sperm from the Cattanach mouse was 4.9%, whereas that of normal mice was 3.3% (Pinkel et al, 1982a). This suggested that the bimodal peaks observed (Figure 2) with flow cytometry represented the X and Y sperm populations.

The difference in genetic sex determination between mammals and birds provided another important clue. In mammals, sex is determined by which spermatozoon fertilizes the ovum, the X- or Y-chromosome–bearing gamete. In avian species, however, sex is determined by the oocyte, not by the fertilizing spermatozoon. Avian species produce only Z sperm. Flow cytometric analyses of cockerel sperm revealed a single peak, whereas those of mammals had two peaks (Garner et al, 1983).

The flow cytometric method was immediately applied to the sperm of domestic animals (Garner et al, 1983).

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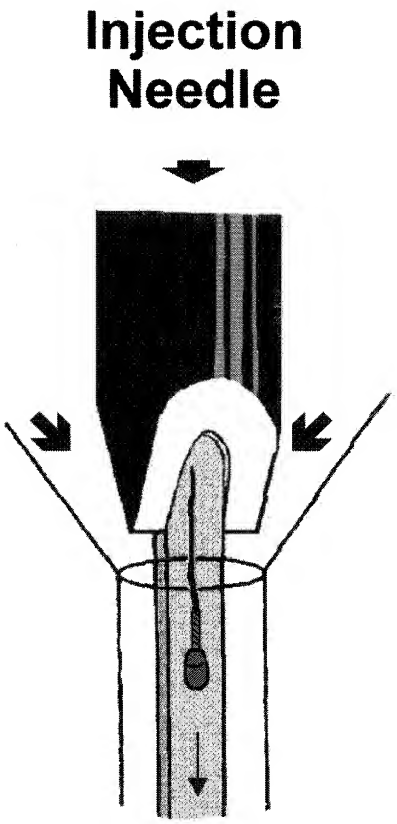


Figure 1. Illustration showing the end of a flattened, beveled, needle injection tube within the nozzle of a flow cytometer. The beveled needle was designed to provide hydrostatic pressure (large arrows), resulting in laminar flow (small arrow). The laminar flow provides increased fluidic pressure so that the flattened oval shape of the sperm head becomes oriented in the laminar stream. This orientation effect increases the proportion of sperm nuclei for which precise DNA measurements can be made. (Fulwyler, 1977; Pinkel et al, 1982a).

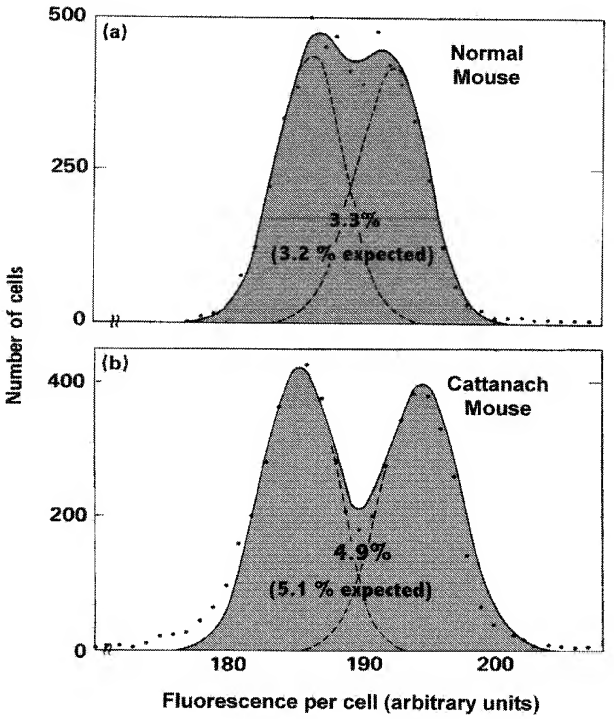


Figure 2. Comparison of the presumptive X and Y sperm populations from normal mouse **(a)** and from a Cattanach male **(b)** with an induced translocation from chromosome 7 to the X chromosome. The expected differences in DNA content between the 2 populations of sperm were 3.2% for normal mice and 5.1% for the Cattanach mouse. Sperm were stained with ethidium bromide and mithramycin, and analyzed by flow cytometry using a beveled needle injection tube to orient the sperm nuclei so that precise DNA measurement could be made. (Adapted from Pinkel et al, 1982a).

The DNA content differences in the bimodal populations of sperm from bulls, boars, rams, and rabbits were 3.9%, 3.7%, 4.0%, and 3.9%, respectively (Garner et al, 1983). The method of preparing sperm for flow cytometric analysis was destructive because the tails and most of the membranes and cytoplasm were removed, leaving the sperm biologically unusable (Gledhill et al, 1984; Gledhill, 1988).

Flow cytometry was also used to test validity of claims that semen had been enriched in either the X or Y sperm by other methods. Using a variety of separation techniques for enrichment, including sedimentation, electrophoresis, Sephadex G-50 filtration, centrifugation, albumin gradients, convection-counterstreaming galvanization, and galvanic and electrical charge differences, semen samples were analyzed to determine if either the X or Y sperm populations had been enhanced. Proportions of X- and Y-chromosome–bearing sperm in 53 samples had not been altered from the expected 50:50 ratio (Garner, 1984; Pinkel et al, 1985).

The first actual separation of mammalian sperm was achieved with *Microtus oregoni,* a vole with a 9% difference in DNA content between its sex-determining sperm (Pinkel et al, 1982b). An explanation for the large DNA difference between the sperm from this vole is that half of the sperm contain a Y chromosome, whereas the other half have no sex chromosome. These sex-chromosome–minus cells are designated "O." Tates et al (1975) identified these two types of unusual sperm in a related European species, *Microtus oeconomus,* using chromosome C-banding techniques. The X chromosome content of somatic cells is reconstituted at fertilization in this species (Pinkel et al, 1982b). This system was not very efficient, but analysis of the sorted nuclei of the microtus "O" sperm indicated that 82% to 95% purity had been obtained, whereas the Y fraction was 72% to 83% pure.

The development of flow cytometric sperm analysis of gametes from domestic species resulted from a collaboration between Lawrence Livermore National Laboratory,

Table 1. *Data from the first study resulting in live offspring from successful sexing of sperm; rabbits were inseminated with flow-sorted X and Y sperm (from Johnson et al, 1989)*

| Treatment of Sperm | Number of Does | | Total Young Born | Percentage and Number of Offspring | | | |
|---|---|---|---|---|---|---|---|
| | | | | Predicted* | | Actual† | |
| | Inseminated | Kindled | | Males, % | Females, % | Males, % (No.) | Females, % (No.) |
| Sorted Y | 16 | 5 | 21 | 81 | 19 | 81 (17) | 19 (4) |
| Sorted X | 14 | 3 | 16 | 14 | 86 | 6 (1) | 94 (15) |
| Recombined X and Y | 17 | 5 | 14 | 50 | 50 | 43 (6) | 57 (8) |

* Predicted from reanalysis of sorted sperm for DNA content relative to X and Y sperm.
† Actual births.

Oklahoma State University, and the United States Department of Agriculture (USDA). This research effort was initiated in 1981 through support by the US Department of Energy, the US Environmental Protection Agency, and a cooperative agreement between Oklahoma State University and the USDA Beltsville Agricultural Research Center.

## Sorting Sperm

The analytical flow cytometric system could identify the X and Y sperm populations, but this technology could not separate living sperm. The analytical system developed at Lawrence Livermore National Laboratory measurement system did not effectively separate intact X and Y sperm. To attain this goal, a Coulter EPICS V laser-based orthogonal flow cytometer/cell sorter at USDA in Beltsville, Maryland, was modified by beveling the sample injection tube tip and substituting a forward angle fluorescence detector and an optical fiber bundle for the light scatter detector so that fluorescence could be measured accurately (Johnson and Pinkel, 1986). This modified instrument was used to sort the first X- and Y-chromosome–bearing sperm nuclei (Johnson et al, 1987b), separating the nuclei into X and Y populations at a purity of 95%. Stoichiometric staining of the sperm nuclei utilized severe treatments with dimethyl sulfoxide (DMSO) washes to remove the surrounding membranes, fixation in ethanol, and protease digestion to ensure availability of DNA to interact with dye (Garner et al, 1983; Johnson et al, 1987b). Chinchilla sperm were the gametes of choice due to a 7.5% difference in DNA content between the X and Y sperm. This sorting procedure also employed a new DNA dye, Hoechst 33342, which stained the DNA of the sperm more efficiently and accurately (Johnson et al, 1987b) than previously used dyes. The Hoechst 33342 staining procedure soon was improved to make the process less damaging to sperm. Sonication of the sample for 10 seconds before staining eliminated the need to prepare the sperm nuclei by exposure to DMSO, ethanol, and proteolysis (Johnson et al, 1987a,b; Johnson and Clarke, 1988). Later it was found that intact sperm could be quantitatively stained with Hoechst 33342 without causing significant damage to cell morphology or motility (Johnson, 1990). The addition of propidium iodide (PI) to the staining mixture provided a means for eliminating dead and damaged sperm from the sorted product. Propidium iodide, which stains membrane-damaged sperm, later was replaced with food coloring (eg, red FD&C #40). This was done because most any food coloring quenches Hoechst 33342 safely, making the signal from Hoechst 33342 bound to DNA different in dead sperm than in live sperm (Johnson et al, 1994, 1999; Rens et al, 1998, 1999; Johnson and Welch, 1999).

## Biological Confirmation of Separation of X and Y Sperm

The report of live rabbit pups produced by flow-sorted sperm (Johnson et al, 1989) was the biological evidence that confirmed the validity of earlier biochemical evidence. The bimodal peaks identified by flow cytometric analysis of DNA content were, in fact, the X and Y sperm populations. The X- and Y-chromosome–bearing rabbit sperm were sorted according to their DNA content and then surgically inseminated into the uteri of rabbits (Johnson et al, 1989). Does inseminated with X-sorted sperm produced litters that were 94% female, whereas those inseminated with Y-sorted sperm produced litters that were 81% male. This demonstrated that phenotypic sex could be predetermined by sorting sperm into X and Y populations prior to insemination (Table 1; Johnson, 1990, 1991, 1992; Johnson et al, 1989).

The efficacy of the sorting procedure was also demonstrated with swine, but the efficiency was lower (Johnson, 1991). Intact boar sperm were sorted into relatively pure populations of Y sperm and inseminated into the isthmus of the oviduct of gilts, resulting in piglets that were 68% male (Johnson, 1992). Analysis of the sorted sperm sample predicted that 80% would be male. Samples sorted for X sperm with a purity of 80% were inseminated, resulting in 74% females. This is the first demonstration in large farm animals that the sex ratio of offspring could be significantly altered.

Table 2. *Pregnancy rates and sex bias from a field study with flow-sorted sperm, in vitro fertilization, cryopreservation of embryos, and embryo transfer (from Cran et al, 1995)*

| Farm | Transfer, No. | Pregnancies, No. | Calved, No. | Aborted, No. | Calves, No. | Males, No. | Females, No. |
|---|---|---|---|---|---|---|---|
| A | 16 | 3 | 3 | 0 | 3 | 3 | 0 |
| B | 20 | 5 | 4 | 1 | 4 | 4 | 0 |
| C | 11 | 5 | 4 | 1 | 4 | 6[a] | 0 |
| D | 2 | 2 | 1 | 1 | 2 | 1 | 1 |
| E | 9 | 3 | 3 | 0 | 3 | 3 | 0 |
| F | 16 | 8 | 8 | 0 | 11 | 11 | 0 |
| G | 7 | 2 | 2 | 0 | 2 | 2 | 0 |
| H | 6 | 4 | 4 | 0 | 4 | 4 | 0 |
| I | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| J | 9 | 3 | 3 | 1 | 4 | 2 | 2 |
| K | 8 | 2 | 2 | 0 | 2 | 1 | 1 |
| Total | 106 | 38 (36%) | 35 (33%) | 4 (11%) | 39 | 37 (90%)[a] | 4 (10%) |

[a] Included twin aborted fetuses.

### *Use of Sex-Sorted Sperm with In Vitro Fertilization*

Sex-sorted bovine sperm were used for in vitro fertilization (IVF) to generate embryos from in vitro–matured oocytes (Cran et al, 1993). Sperm were sorted using a modified Becton Dickinson FACStar Plus flow cytometer/cell sorter. The sperm were stained with Hoechst 33342 and sorted at a rate of 100 cells/s to obtain an X sperm population of 79% purity and a Y population of 70% purity (Cran et al, 1993). Twin transfers of 9 sex-selected embryos produced 4 pregnancies, resulting in 3 males and 3 female calves (Cran et al, 1993), all of the projected sex. These births demonstrated that sex-sorted bovine sperm could result in living offspring. Further studies revealed that a sex ratio of 90% male could be achieved (Cran et al, 1995; Table 2). The skewed sex ratio of the calves born from these transfers was consistent with the predetermined sex as determined by the polymerase chain reaction assay of excised cells from similarly selected blastocysts (Cran et al, 1993, 1995). These findings set in motion the potential commercialization of the sorting and IVF technology when the USDA issued a license to Mastercalf, Ltd of the United Kingdom.

### *Low-Dose Insemination*

Flow sorting had improved by 1996 so that it was possible to produce enough living sexed sperm that oviductal insemination, IVF, or intracytoplasmic sperm injection (ICSI) was feasible. The numbers of sperm available, however, were insufficient for routine artificial insemination because normal practice for cattle used on the order of $20 \times 10^6$ sperm/dose. This number seems unreasonable considering that only one sperm is needed to fertilize an oocyte. The problems associated with sperm successfully traversing the female tract of the sow was bypassed by inseminating sorted porcine sperm directly into the isthmus of the oviduct (Johnson, 1992). The likelihood of placing fewer sperm at a site closer to where fertilization takes place in the cow led to an experiment to test the concept that fewer sperm per insemination dose could be used if the gametes were placed more optimally within the female tract. This was tested by depositing only $1–5 \times 10^5$ sperm/dose deep into the uterine horn of heifers using the atraumatic, side-opening sheaths commonly employed in embryo transfer (Seidel et al, 1996a,b; 1997). Under some conditions, reasonable pregnancy rates were achieved with this procedure.

This low-dose advance was coupled with sex-sorted bovine sperm (Seidel et al, 1997). In the initial study, semen was collected in Pennsylvania, sex-sorted in Maryland, and shipped by air to Colorado for deep uterine insemination of $1–2 \times 10^5$ unfrozen, sexed sperm. Even with this long-distance approach, 17 calves were produced, 14 of 17 which were of the predicted sex (Seidel et al, 1997).

These early pregnancies from shipped, sex-sorted sperm were followed by formation of XY, Inc, and initiated the installation of a high-performance sperm sorter in Colorado so that it would be within reasonable driving distance from cattle to be inseminated. The new instrument could sort 500–600 live sperm per second. Sufficient X-selected sperm were prepared so that 35 heifers could be inseminated with $3 \times 10^5$ unfrozen sperm/dose. Forty-two percent of the heifers inseminated with the sexed sperm became pregnant compared with a 54% pregnancy rate for controls inseminated with $3 \times 10^5$ motile unfrozen sperm (Seidel et al, 1998). Eighteen of the 19 calves born to X-sorted sperm were females (Seidel et al, 1998). Pregnancy rates were 80% of what was achieved with unsexed sperm.

### *Cryopreservation of Sorted Sperm*

Success with sexed sperm maintained at near 5°C was followed by an attempt to cryopreserve sorted cells at $1 \times 10^6$ sperm per dose in 0.25-mL straws (Schenk et al,

1999). This was twice the concentration that had been used successfully for unfrozen, sexed sperm to compensate for cell death due to cryopreservation and thawing. After sorting, sperm were concentrated by centrifugation to about 80 × $10^6$/mL so that they could be reconstituted to 20 × $10^6$/mL and placed into 0.25-mL straws. Pregnancy rates with sexed, cryopreserved sperm were identical, 52%, to that achieved earlier with a third more as many unfrozen sperm (Seidel, 1999a,b; Schenk et al, 1999). With this advance, it became obvious that sperm-sorting facilities should be located adjacent to semen collection sites (Amann, 2000) so that sexed sperm could be cryopreserved. The cyropreservation of sexed sperm allows much greater flexibility in distribution of the product for insemination. Although the procedures for sexing sperm result in slightly lower post-thaw motilities and acrosomal integrities compared with control sperm, this damage is minor compared with that caused by routine cryopreservation (Amann, 2000).

The numbers of sex-sorted, cryopreserved sperm used for insemination have ranged from 1 to 6 × $10^6$ sperm/straw (Seidel et al, 1999c). Normal insemination doses usually are 10–20 × $10^6$ sperm. Sperm now can be sorted at rates approaching 4000 per second of each sex, but it is advantageous to use as few sperm per insemination as necessary to achieve acceptable pregnancy rates.

### *Sperm Sorting Speed*

Sorting speed is dependent on orientation of the sperm through the instrument. An improved system for orienting sperm during the sorting process was developed at USDA (Rens et al, 1998, 1999; Johnson et al, 1999; Welch and Johnson, 1999; Johnson, 2000). This nozzle design increased the proportion of sperm that could be correctly measured from 25% to more that 60% (Rens et al, 1998, 1999). The new nozzle was adapted for a high-speed sorter and increased sorting rates from 2 to 6 × $10^6$ sperm/h for each sex (Rens et al, 1998; Johnson et al, 1999). The USDA sperm-orienting nozzle was adapted for use with Cytomations SX MoFLo (Cytomations, Fort Collins, Colo) and its orienting ability enhanced by modifications developed at XY Inc (Figure 3). Implementation of these and other modifications to the SX MoFlo by XY Inc have significantly increased the efficiency of sorting X and Y sperm. This modified instrument is capable of sorting 15 × $10^6$ bovine sperm/h for each sex (Seidel, 2000; Schenk, 2001).

### *Pregnancy and Calving Rates*

Optimal use of sexing technology requires a level of management that is more intensive than some livestock operations because application of the current technology requires careful timing of inseminations (Seidel et al, 1999a,b,c). Reasonable pregnancy rates have been

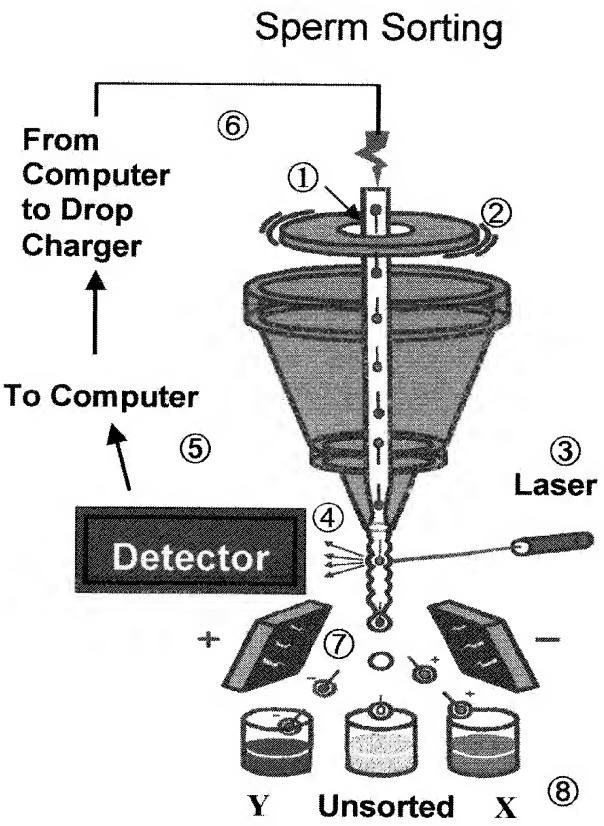


Figure 3. Flow Cytometer/sperm sorter system. The sperm are pumped through the system after having been stained with a DNA-binding dye (1). A piezoelectric crystal vibrator (2) causes 90 000 droplets to form each second as the stream exits the system. A UV laser illuminates the sperm as they flow by the beam (3). X sperm fluoresce with 4% more intensity than Y sperm (4). The signal detected with a photomultiplier tube is sent to a computer that processes the fluorescence detected and categorizes sperm as X, Y, or uncertain (5). Negative, positive, or no charge is applied to droplets as they emerge from the stream (6). As the charged droplets pass between continuously charged plates, they are deflected (7). Sperm are collected in 3 containers: X, Y, uncertain or no sperm (8).

achieved with low-dose sexed, cryopreserved sperm in heifers, but little work had been done with lactating cows (Seidel et al, 1999a,b,c). Under current practices, the ability to cryopreserve sex-sorted sperm successfully has allowed flexibility for use in breeding estrus-synchronized or unsynchronized heifers.

There has been very little difference in pregnancy rate after inseminations of 1.0 to 1.5 × $10^6$ vs. 3.0 × $10^6$ sexed, cryopreserved sperm based on more than 1000 inseminations (Seidel et al, 1999a; Table 3). In some recent trials, pregnancy rates for sexed, cryopreserved sperm have been 90% of control heifers inseminated with 7 to 20 times more sperm/dose. Relative to the site of insemination, in only 1 of 11 trials did inseminations into the uterine horns result in higher pregnancy rates than when

Table 3. *Summary of 11 field trials conducted at Colorado State University from 1997–1999 with sexed sperm showing pregnancy results from insemination of 965 heifers with sexed sperm and 404 with unsexed control semen from the same bulls (from Seidel et al, 1999)*

| Trials | Sperm No./Site | Heifers, No. | Pregnant (%) |
|---|---|---|---|
| 5, 6, 9, 11 | 1.0–1.5 × $10^6$/body | 77 | 36 (47) |
| | 3.0 × $10^6$/body | 76 | 38 (50) |
| | 1.0–1.5 × $10^6$/horn | 72 | 32 (44) |
| | 3.0 × $10^6$/horn | 72 | 39 (54) |
| | 20 × $10^6$/body, control | 93 | 61 (66) |
| 4–6, 9–11 | 1.0–1.5 × $10^6$/body | 176 | 98 (56) |
| | 3.0 × $10^6$/body | 171 | 88 (51) |
| | 20 × $10^6$/body, control | 183 | 124 (68) |
| 5–7, 9, 11 | 1.0–1.5 × $10^6$/body | 163 | 70 (43) |
| | 3.0 × $10^6$/horn | 158 | 85 (54) |
| | 20 × $10^6$/body, control | 128 | 79 (62) |

the sperm were placed in the uterine body (Seidel et al, 1999c).

### Normality of Offspring

More than 1000 live births from sexed sperm have been produced in 6 species with no gross abnormalities being observed (Seidel et al, 1999a,b; Cran, 2000). Although offspring born from pregnancies generated with sexed sperm appear normal from a general phenotypic standpoint, rigorous epidemiological studies need to be conducted to verify and strengthen these observations. The concern is that the process of sexing sperm could damage the sperm DNA and, thus, could increase the incidence of genetic abnormalities. Nearly 1000 heifers have been inseminated with sexed sperm and their subsequent histories noted. Many more are currently gestating. No increase in embryonic death between 1 and 2 months of gestation has been detected, with very few abortions occurring between 2 months of gestation and calving (Seidel et al, 1999c).

### Commercialization

All prior attempts to develop sexed sperm as a commercial product have ended in failure. This basic biological problem is highly attractive from both conceptual and monetary standpoints. However, potential differences between the X and Y sperm are relatively small and are not evident except for DNA. A variety of simplistic to very complicated methods were tried before the first repeatable success was reported in 1989 using flow-sorting for sexing sperm (Johnson et al, 1989). This sexing procedure, which was originally developed for living sperm at the USDA Beltsville Agricultural Research Center by Larry Johnson (Johnson et al, 1989), was patented by USDA. The first commercial license was issued to Mastercalf Ltd, and this group demonstrated that sexed calves could be produced by coupling the sperm sexing procedure with IVF and transferring the resulting embryos to recipients (Cran et al, 1993, 1995). This approach, however, never became commercially viable.

It is estimated that nearly $20 million has been invested in developing this technology from conceptualization in the early 1980s to the recent commercialization effort in 2000. Although other approaches are currently being pursued (Johnson and Seidel, 1999), the only verified method for selecting sex offspring is flow cytometric sorting of sperm relative to DNA content. This sperm-sexing technology, which was patented by USDA (U.S. patent 5 135 759), has been licensed by USDA for all nonhuman mammals through the Colorado State University Research Foundation, to XY Inc, a private company.

### Summary

Sperm sexing can be used to produce sexed offspring with 85%–95% accuracy (Amann, 1999; Johnson and Seidel, 1999; Seidel et al, 1999a). On September 1, 2000, the sale of sexed bovine sperm commenced in the United Kingdom. It will be interesting to see to what degree sexed sperm penetrate the semen market. This verified sexed product sets the stage for commercialization around the world in major animal producing countries. This commercialization of sexed sperm occurred nearly 20 years after technology for accurately determining the proportion of X and Y sperm in semen was first developed at Lawrence Livermore National Laboratory. It came about due to advances in both the hardware and software components of computer science, biophysics, cell biology, and applied reproductive physiology plus efforts of innovative scientists. Many individuals have contributed to making semen sexing in animals a commercial reality since the research team of Bart Gledhill, Dan Pinkel, Duane Garner, Susan Lake, and Larry Johnson began following up on the first flow cytometric studies on human sperm by Friedrich Otto, Wolfgang Göhde, and Marvin Meistrich. There was also major input from personnel at USDA Beltsville Agricultural Research Center as well as scientists at Cambridge University, Atlantic Breeders Cooperative, Colorado State University, and XY Inc. These include Chuck Allen, Rupert Amann, David Cran, Patrick Doyle, Mike Evans, Lisa Herickhoff, Mervyn Jacobson, Kehuan Lu, Chris Polge, Wim Rens, John Schenk, George Seidel, Glenn Welch, and many others.

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# Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting[1]

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**ABSTRACT:** The objectives of this study were to determine whether calves produced by sexed sperm differed from controls and to what extent the sex ratio of calves was altered by the sexing procedure. Data were collected from 1,169 calves produced from sperm sexed by flow cytometry/cell sorting after staining with Hoechst 33342, and 793 calves produced from control sperm during breeding trials between 1997 and 2001. Least squares ANOVA were completed using factors of treatment (sexed vs. control sperm), 19 management groups from 13 field trials, and calf sex. Responses analyzed include gestation length, birth weight, calving ease, calf vigor, weaning weight, abortion rate, and death rates (neonatal and through weaning). No significant difference was observed for any response due to treatment or treatment interactions ($P > 0.10$). Therefore, calves produced from sexed sperm grew and developed normally both pre- and postnatally. A neurological disorder was observed in four control calves and one sexed calf from one farm. No gross anatomical abnormalities were reported for any calves in the study. Differences were observed for all responses among management groups ($P < 0.03$ for abortions and $P < 0.01$ for all other responses). Heifer and bull calves differed ($P < 0.001$) in gestation length (278.4 and 279.6 d), birth weight (32.8 and 35.2 kg), calving ease (1.15 and 1.30), and weaning weight (233 and 247 kg). Gestation length did not affect characteristics of calves. The sex ratio at birth of calves from unsexed control sperm was 49.2% male. Sexing accuracy of X-sorted sperm was 87.8% female calves, and Y-sorted sperm produced 92.1% male calves. Flow cytometry/cell sorting can be used to preselect sex of calves safely with approximately 90% accuracy.

Key Words: Calves, Flow Cytometry, Normality, Sexing, Sex Ratio, Sperm

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## Introduction

Flow cytometry/cell sorting can be used to measure DNA content of sperm that have been stained with the fluorescent DNA binding dye, Hoechst 33342 (**H33342**), in order to separate populations of X- or Y-chromosome-bearing sperm (Johnson et al., 1999). This process has produced offspring with significantly skewed sex ratios of the desired sex in several species (Seidel and Garner, 2002). Sex preselection is of interest to cattle producers. For example, X-sorted sperm can produce replacement heifers from more selected, genetically superior dams, whereas Y-sorted sperm can produce bull calves to be sold for slaughter from less valuable dams (Hohenboken, 1999; Seidel, 2002). The efficiency of progeny testing dairy bulls would be improved by using X sperm to produce the required heifers from the fewest dams (Hohenboken, 1999; Seidel, 2002).

The sexing process damages sperm in several ways. Motility, viability, and membrane integrity are compromised by high rates of dilution as protective molecules are removed (Catt et al., 1997; Maxwell and Johnson, 1997). Physical stress during sorting and handling of sperm leads to membrane damage, including premature acrosome reactions (Maxwell et al., 1998). Hoechst 33342 has caused chromosomal damage under some conditions (Libbus et al., 1987). High pressure within the system has been associated with decreased fertility (Seidel et al., 2003). No increase in calf abnormalities has been reported to date with sexed sperm relative to controls. There is a hint, how-

[1]Sperm were sorted by personnel at XY, Inc. Numerous persons assisted with estrus detection, artificial insemination, and pregnancy and fetal sex diagnoses. We especially acknowledge personnel at the farms and ranches who allowed us to use their cattle and assisted with data collection. This research was supported financially by XY, Inc., Fort Collins, CO, and the Agric. Exp. Stn. at Colorado State University.

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ever, that early embryonic mortality may be increased slightly with sexed sperm (Seidel et al., 1999).

Many offspring have been produced from sexed sperm, but no thorough study of the normality of offspring has been reported. Therefore, characteristics of calves were studied to determine whether there are any differences between populations of calves from sexed vs. unsexed sperm.

## Materials and Methods

### *Sorting and Preparation of Sperm*

Semen was collected from 30 bulls using an artificial vagina and sorted into X- and Y-chromosome populations using flow cytometry/cell sorting as described by Seidel and Garner (2002). Briefly, semen was stored undiluted at 20 to 23°C for up to 8 h before sorting, during which time, sequential aliquots of sperm were prepared for sorting every 90 min. Sperm were stained in a modified Tyrode's medium supplemented with albumin, lactate, and pyruvate, to which H33342 (Sigma Chemical Co., St. Louis, MO) was added followed by addition of red food dye. Red food dye was used to identify dead sperm because it quenches fluorescence of the bound H33342 in dead sperm (Schenk et al., 1999), which can then be discarded. A Tris-based sheath fluid surrounded the sorting medium as sperm passed through the sorter (Schenk et al., 1999) at speeds up to 90 km/h. During this process, sperm were diluted an additional 10-fold.

An argon laser was used to excite the H33342 bound to the DNA of the sperm. Fluorescence detectors at a 90° angle to each other measured the fluorescence signals of the sperm to allow separation based on the difference in DNA content between the X- and Y-chromosomes—approximately 3.8% in cattle. The signal at 90° was used to determine which sperm were properly oriented for accurate evaluation, whereas the signal at 0° was used to determine DNA content (Seidel and Garner, 2002). Signals were then processed, and droplets were electrically charged depending on the presence of X- or Y-chromosome-bearing sperm. Charged droplets were deflected using charged plates (Seidel and Garner, 2002), and the sorted streams were collected into tubes with an egg yolk-Tris extender (Schenk et al., 1999; Seidel and Garner, 2002). Semen was concentrated by centrifugation and then extended and packaged in 0.25-mL straws for cryopreservation in liquid nitrogen vapor. In several of the early trials (Seidel et al., 1999; Schenk et al., 1999), sperm were not frozen before AI. Control semen was extended and cryopreserved from the same ejaculates as the sexed sperm, but was not subjected to staining or other procedures applied to the sexed sperm other than holding so that control and sexed sperm were frozen at the same time.

### *Insemination of Heifers and Cows*

Groups of heifers and cows of beef breeds, mostly Angus and one group of Holstein heifers, were inseminated with sexed or control sperm during field trials from 1997 to 2001 (Table 1). Estrus was synchronized using one of three methods (Table 1) as described by Seidel et al. (1999). The first method was daily feeding of 0.5 mg of melengestrol acetate (**MGA**) for 14 d followed by 25 mg of $PGF_{2\alpha}$ injected i.m. 17 to 19 d later. The second method used two doses of 25 mg of $PGF_{2\alpha}$ injected i.m. 12 d apart. The third method used 50 or 100 μg of GnRH injected i.m. followed by 25 mg of $PGF_{2\alpha}$ injected i.m. 7 d after GnRH treatment.

Animals were visually observed every 12 h to identify those in estrus. Inseminations took place in the evening, 12 or 24 h following initial observation of estrus. Cooled liquid semen was used in several early trials (Table 1). About two-thirds of the inseminations in each trial were with sexed sperm, and unsexed control sperm were used for the remaining inseminations. Inseminations were done as 13 different field trials in 13 herds with various experimental designs, mostly concerning numbers of sperm per inseminate and site of insemination (Seidel et al., 1999). However, after pregnancy diagnosis, three of the herds were divided into three groups, giving 19 management groups by calving time.

### *Pregnancy Diagnosis and Management*

Transrectal ultrasonography was used to determine pregnancy status and fetal sex approximately 2 mo after insemination for all trials except Trial 13. After pregnancy diagnosis, heifers and cows were managed at the various farms with different levels of management through calving and weaning.

### *Collection of Calf Data*

Forms requesting calving data were distributed to the respective farms during the calving season. The requested information included birth date, birth weight, calf sex, calving ease (1 = no assistance, 2 = some assistance, 3 = difficult pull, 4 = delivery by caesarian section), calf vigor (1 = healthy, nursed immediately, 2 = took some time to nurse, 3 = assistance to suckle, 4 = died shortly after birth, 5 = dead at birth; calves that died shortly after birth were scored as 4 even if they had reasonable vigor just after birth), and weaning weight. Observed abortions and deaths were recorded, as was any information on obvious abnormalities. Farm personnel were contacted to clarify any missing or unclear data. When necessary, visits were made to farms to complete datasets. Some farms did not collect all requested data, such as birth and weaning weights. Animals from those farms were not included in statistical analyses of those responses. Data were analyzed for 1,169 sexed and 793 control calves.

**Table 1.** Characteristics of trials

| Trial[a] | Location | Year | Breed | Heifers or cows | Bulls | No. of calves | Synchronization agent[b] |
|---|---|---|---|---|---|---|---|
| 1 | Wyoming | 1997 | Angus | Heifers | 5201, 6259, 6372 | 48 | MGA, $PGF_{2\alpha}$ |
| 2 | Wyoming | 1998 | Angus | Heifers | 5201, 7030, 7102, AN523 | 54 | MGA, $PGF_{2\alpha}$ |
| 3 | Wyoming | 1998 | Angus | Cows | 5201, 7015, 7102, AN523 | 80 | GnRH, $PGF_{2\alpha}$ |
| 4 | Wyoming | 1999 | Angus | Heifers | AN004, AN007, AN008 | 66 | GnRH, $PGF_{2\alpha}$ |
| 5 | Wyoming | 1999 | Angus | Cows | AN004, AN005, AN006 | 55 | GnRH, $PGF_{2\alpha}$, GnRH |
| 6 | Colorado | 1999–2000 | Holstein | Heifers | H002, H003, H008 | 227 | $PGF_{2\alpha}$, $PGF_{2\alpha}$ |
| 7A | Colorado | 2000 | Angus | Heifers | AN012, AN013 | 65 | MGA, $PGF_{2\alpha}$ |
| 7B | Colorado | 2000 | Angus | Heifers | AN012, AN013 | 88 | MGA, $PGF_{2\alpha}$ |
| 7C | New Mexico | 2000 | Angus | Heifers | AN012, AN013 | 48 | MGA, $PGF_{2\alpha}$ |
| 8A | Nebraska | 2000 | Red Angus | Heifers | AR002, AR004 | 54 | MGA, $PGF_{2\alpha}$ |
| 8B | Nebraska | 2000 | Red Angus | Heifers | AR002, AR004 | 49 | MGA, $PGF_{2\alpha}$ |
| 8C | Nebraska | 2000 | Red Angus | Heifers | AR002, AR004 | 67 | MGA, $PGF_{2\alpha}$ |
| 9 | Wyoming | 2000 | Angus | Heifers | AN16, AN18, AN19 | 49 | MGA, $PGF_{2\alpha}$ |
| 10 | Wyoming | 2001 | Angus | Cows | AN010, AN023, AN028 | 78 | MGA, $PGF_{2\alpha}$, and GnRH, $PGF_{2\alpha}$ |
| 11A | Colorado | 2001 | Angus | Heifers | AN25, AN26, AN27 | 49 | MGA, $PGF_{2\alpha}$ |
| 11B | Colorado | 2001 | Angus | Heifers | AN25, AN26, AN27 | 101 | MGA, $PGF_{2\alpha}$ |
| 11C | New Mexico | 2001 | Angus | Heifers | AN25, AN26, AN27 | 51 | MGA, $PGF_{2\alpha}$ |
| 12 | Colorado | 2001 | Angus | Heifers | AN25, AN27 | 80 | GnRH, $PGF_{2\alpha}$ |
| 13 | Wyoming | 2001 | Angus crosses | Heifers | AR011, AR012 | 653 | None |

[a]For Trials 4 to 13, only frozen semen was used.
[b]MGA = melengestrol acetate.

*Data Editing*

Data were edited to ensure, as much as possible, that all calves included in the analysis were in fact calves resulting from the sexed-semen experiment. Editing also ensured complete data for essentially all calves included in the analysis, and editing was done identically for calves resulting from sexed or control sperm. For calves with long gestation lengths (>290 d; i.e., born well after the expected calving date), breeding records were checked to determine whether pregnancy was from sexed AI or from possible rebreeding. Those rebred were excluded from the study. There were 61 calves excluded from the study because of excessively long gestation length; these calves were born in the absence of rebreeding records. Short gestation lengths (range 255 to 258 d) included six premature births (five of which died) and two dead fetuses aborted 188 d postinsemination. There were 79 additional abortions deduced when heifers were pregnant at 2 mo of gestation and then open after summer pasture. Loss of animals or records accounted for 29 animals being excluded from the study; further calving information was therefore not available for these animals. Twins (10 sets; six sexed and four control) were not included in the analysis due to differences in gestation length, birth weight, and weaning weight associated with twins compared with single-birth calves.

*Data Analysis*

After compilation and editing of calving information, data were analyzed within each trial group (herd), as well as in a combined analysis of calves from all trials to determine whether any differences existed between the sexed and control populations. Factorial, least squares ANOVA with Type III sums of squares were performed using the GLM procedures of the Statistical Analysis System (SAS Inst., Inc., Cary, NC). Data expressed as a percentage were transformed using the arc sine transformation. Factors used in the analyses include farm (management group), sex of calf, sexed vs. control semen, and all first-order interactions; gestation length was included as a covariate in some cases. Management group was considered to be a random effect and the other factors were fixed effects. Responses analyzed include gestation length, birth weight, calving ease, calf vigor, weaning weight, abortion rates, and death rates neonatally and through weaning. Adjusted weaning weights were not analyzed because they were not available for a majority of the calves. Results of analyses for each of the 19 individual management groups are not presented because there rarely were statistically significant effects due to the relatively low numbers of animals within each herd.

In addition to the aforementioned analyses, data from individual trial groups were analyzed to determine possible effects of bull, site of insemination, and sperm doses. However, none of the factors in these

**Table 2.** Analysis of variance for gestation length

| Source | DF | Mean square | *F*-value | Significance |
|---|---|---|---|---|
| Group | 18 | 194.59 | 10.63 | <0.0001 |
| Treatment | 1 | 1.12 | 0.07 | 0.8047 |
| Calf sex | 1 | 308.02 | 22.29 | <0.0001 |
| Group × treatment | 18 | 15.97 | 0.87 | 0.6131 |
| Group × calf sex | 18 | 13.82 | 0.76 | 0.7545 |
| Treatment × calf sex | 1 | 0.05 | 0.00 | 0.9583 |
| Error | 1,887 | 18.3 | | |

**Table 3.** Least squares means for characteristics for treatment

| Treatment | Gestation length, d | Birth wt, kg | Calving ease[a] | Calf vigor[b] | Weaning wt, kg | Abortion rate, % | Neonatal death rate, % | Total death rate, % |
|---|---|---|---|---|---|---|---|---|
| Control | 278.9 | 34.1 | 1.23 | 1.10 | 241.2 | 5.0 | 4.0 | 8.5 |
| No. | 787 | 673 | 507 | 262 | 348 | 560 | 787 | 787 |
| Sexed | 279.0 | 33.9 | 1.22 | 1.17 | 238.9 | 4.5 | 3.5 | 8.3 |
| No. | 1,158 | 923 | 740 | 463 | 454 | 829 | 1,158 | 1,157 |
| Pooled SD[c] | 4.28 | 4.33 | 0.52 | 0.64 | 22.8 | 2.3 | 2.1 | 2.7 |

[a]1 = no assistance; 2 = some assistance; 3 = difficult pull; and 4 = delivery by caesarian section.
[b]1 = healthy, nursed immediately; 2 = took some time to nurse; 3 = assistance to suckle; 4 = died shortly after birth; 5 = dead at birth. Calves that died shortly after birth were scored as 4 even if they had reasonable vigor just after birth.
[c]Square root of the error term of the ANOVA.

analyses had any significant ($P > 0.10$) effect on characteristics of calves or pregnancies, so related data are not presented.

The accuracy of sperm sexing and fetal sex diagnosis by ultrasound were calculated as the percentage of correct sorted sex out of the total sexed. Sex ratios for each trial were calculated as the percentage of males out of the total number of calves for sexed, control, and combined populations.

## Results and Discussion

### *Gestation Length*

The ANOVA for gestation length is shown in Table 2. The ANOVA for other responses were similar in format to gestation length, and therefore are not presented. Differences in gestation length were observed only for management group and calf sex ($P < 0.001$; Table 2). Least squares mean gestation lengths for sexed and control calves were nearly identical ($P = 0.80$; Table 3). Therefore, the sexing process did not influence gestation length.

Bull calves had a 1.2 d longer ($P < 0.001$) gestation length than heifer calves (Table 4). Sex of calf has previously been shown to influence gestation, with bulls routinely having longer gestation lengths than heifers (Azzam and Nielsen, 1987; Reynolds et al., 1990).

Least squares means for gestation length by management group ranged from 276.8 to 282.1 d (Table 5). Groups were managed in different environmental conditions, including different climates, diets (availability of pasture, etc.), and level of management. Management group differences also included animal differences, such as breed and age of dam (heifer vs. cow) as well as breed of bull. Age of dam (Bourdon and Brinks, 1982), parity of dam (Foote, 1981; Azzam and Nielsen, 1987), and breed of bull (Reynolds et al., 1990) have all been reported to influence gestation length.

### *Birth Weight*

Birth weights were obtained for 1,596 calves. Groups in which birth weight was not collected (6, 8A, 8B) were not included in this analysis. Differences were observed for management groups and calf sex (Tables 4 and 5; $P < 0.001$). Sexed and control treatments showed no difference ($P = 0.48$; Table 3). No interactions were observed among the factors ($P > 0.10$).

Numerous factors influencing birth weight, such as age and breed of dam and sire effects and environmental factors (e.g., weather and feed availability), likely contributed to the management group differences. Age of dam influences birth weight, with younger dams producing lighter calves (Reynolds et al., 1990; Rege

**Table 4.** Least squares means for characteristics for calf sex

| Calf sex | Gestation length, d | Birth wt, kg | Calving ease[a] | Calf vigor[b] | Weaning wt, kg | Abortion rate, % | Neonatal death rate, % | Total death rate, % |
|---|---|---|---|---|---|---|---|---|
| Female | 278.4[c] | 32.8[c] | 1.15[c] | 1.16 | 233.5[c] | 2.8 | 4.5 | 7.8 |
| No. | 1,250 | 987 | 756 | 428 | 440 | 643 | 1,250 | 1,250 |
| Male | 279.6[d] | 35.2[d] | 1.30[d] | 1.10 | 246.6[d] | 4.7 | 3.0 | 8.9 |
| No. | 695 | 609 | 491 | 297 | 362 | 341 | 695 | 694 |
| Pooled SD[e] | 4.28 | 4.33 | 0.52 | 0.64 | 22.8 | 2.0 | 2.1 | 2.7 |

[a]1 = no assistance; 2 = some assistance; 3 = difficult pull; and 4 = delivery by caesarian section.
[b]1 = healthy, nursed immediately; 2 = took some time to nurse; 3 = assistance to suckle; 4 = died shortly after birth; 5 = dead at birth. Calves that died shortly after birth were scored as 4 even if they had reasonable vigor just after birth.
[c,d]Means differ, $P < 0.001$.
[e]Square root of the error term of the ANOVA.

**Table 5.** Least squares means for characteristics for each management group[a]

| Group | Gestation length, d | Birth wt, kg | Calving ease[b] | Calf vigor[c] | Weaning wt, kg | Abortion rate, % | Neonatal death rate, % | Total death rate, % |
|---|---|---|---|---|---|---|---|---|
| 1 | 276.9 | 35.64 | 1.70 | — | 245.6 | 0.0 | 1.0 | 12.0 |
| 2 | 278.6 | 35.86 | 1.69 | — | 224.4 | 3.7 | 2.0 | 9.0 |
| 3 | 277.8 | 38.07 | 1.02 | — | 226.9 | 6.3 | 0.0 | 8.0 |
| 4 | 276.8 | 33.69 | 1.29 | 1.13 | 222.4 | 2.0 | 2.0 | 8.0 |
| 5 | 278.1 | 37.32 | 0.99 | 1.13 | 228.9 | 5.5 | 3.0 | 5.0 |
| 6 | 277.1 | — | 1.60 | 1.21 | — | 7.6 | 17.0 | 18.0 |
| 7A | 279.8 | 31.71 | 1.09 | 1.16 | 259.2 | 7.6 | 6.0 | 7.0 |
| 7B | 281.0 | 33.77 | 1.08 | — | 270.2 | 6.7 | 4.0 | 10.0 |
| 7C | 280.5 | 30.86 | — | — | 257.3 | 0.0 | 0.0 | 0.0 |
| 8A | 282.8 | — | 1.25 | — | — | 0.0 | 0.0 | 0.0 |
| 8B | 281.3 | — | 1.23 | — | — | 3.3 | 2.0 | 9.0 |
| 8C | 276.1 | 30.30 | 1.20 | — | 228.3 | 5.3 | 7.0 | 15.0 |
| 9 | 278.1 | 33.40 | 1.45 | 1.38 | 228.1 | 0.0 | 10.0 | 14.0 |
| 10 | 280.2 | 39.90 | 1.00 | 1.06 | 219.2 | 6.0 | 1.0 | 8.0 |
| 11A | 278.4 | 32.35 | 1.08 | 0.99 | 272.0 | 7.3 | 6.0 | 14.0 |
| 11B | 279.5 | 32.55 | 1.01 | 1.08 | 225.1 | 10.2 | 2.0 | 6.0 |
| 11C | 281.2 | 30.72 | 1.08 | 1.05 | 253.3 | 1.9 | 4.0 | 8.0 |
| 12 | 276.7 | 32.79 | 1.09 | — | — | 12.7 | 0.0 | 4.0 |
| 13 | 279.5 | 34.76 | — | — | — | — | 5.0 | — |
| Pooled SD[d] | 4.28 | 4.33 | 0.52 | 0.64 | 22.8 | — | — | — |

[a]All responses differed among groups by ANOVA, $P < 0.001$ for all responses except abortion rate, $P < 0.03$, and calf vigor, $P < 0.01$.

[b]1 = no assistance; 2 = some assistance; 3 = difficult pull; and 4 = delivery by caesarian section.

[c]1 = healthy, nursed immediately; 2 = took some time to nurse; 3 = assistance to suckle; 4 = died shortly after birth; 5 = dead at birth. Calves that died shortly after birth were scored as 4 even if they had reasonable vigor just after birth.

[d]Square root of the error term of the ANOVA.

and Famula, 1993; Holland and Odde, 1992). The least squares means in Table 5 show higher birth weights in calves born to multiparous cows (Groups 3, 5, and 10) compared with primiparous heifers. Dam weight also influences birth weight, with heavier dams producing heavier calves (Holland and Odde, 1992). There is also great variability in birth weight among breeds (Holland and Odde, 1992). Large-breed sires also usually produce calves of higher birth weight (Reynolds et al., 1990).

Lower birth weights were observed at several ranches where there were concerns about feed availability. Locoweed was prominent at one location (Group 7C), whereas another group (8C) had a feed problem suspected of causing abortion in several heifers. Birth weights in Wyoming appeared to be higher than in Colorado, likely due to differences in genetic selection. Season influences birth weight as well, as spring calves usually are heavier than fall calves (Rege and Famula, 1993). Most calves in this study were born in the spring. Trial 6 took place over 1.5 yr, with calves born in different seasons, but birth weights were not available at this location.

Bull calves were heavier than heifer calves at birth ($P < 0.001$; Table 4). Previous studies also demonstrated higher birth weights for bull calves (Gregory et al., 1991; Holland and Odde, 1992). This higher birth weight in bulls may be due to longer gestation length and/or presence of androgens in the male fetus (Holland and Odde, 1992).

### *Calving Ease*

Calving ease scores were recorded for only 1,247 calves because two farms (i.e., farms 7C and 13) did not collect these data and were not included in the analysis. Differences were observed among management groups and for calf sex ($P < 0.001$). In agreement with other studies, bull calves resulted in more dystocia than heifers (Gregory et al., 1991; Bellows et al., 1996). The larger size of bull calves at birth is probably responsible for their higher mean calving ease scores compared with heifers (Table 4). Least squares mean calving ease scores (Table 3) were similar for sexed and control calves ($P = 0.87$).

Mean calving ease scores for management groups ranged from 0.99 to 1.70 (Table 5). Differences observed in management groups might be associated with dam, bull, and calf factors that influence dystocia. Trials involving cows (Groups 3, 5, and 10) show lower mean calving ease scores than many of the trials involving heifers (1, 2, 4, 6, and 8). This is in agreement with other findings that younger dams have higher average calving ease scores than older dams (Reynolds et al., 1990).

### *Calf Vigor*

Calf vigor scores were collected in nine management groups (Table 5), from 725 calves. There were significant differences among management groups ($P < 0.01$),

likely due in part to the subjective nature of such scoring and partly due to differences in cow vs. heifer dams. However, there were no significant differences in calf vigor scores due to sexed vs. control semen, nor due to sex of calf or interactions ($P > 0.10$; Tables 3 and 4).

### *Weaning Weight*

Weaning weights were not collected at all farms; calves for which no weaning weight was recorded were not analyzed. Differences were observed between management groups and calf sex ($P < 0.001$), whereas no difference was observed between treatments of sexed and control sperm ($P = 0.24$).

The larger size of bull calves at birth contributes to their greater weaning weight. These differences are apparent in the least squares means observed for weaning weights in bull and heifer calves (Table 4). Bull calves often grow faster than heifer calves, but this can be influenced by environment. Reynolds et al. (1990) showed that during some years, females grew faster than males, depending on environmental conditions. An interaction between management group and sex of calf was observed for weaning weight in this study ($P < 0.00$). This may be caused by different genetics between management groups.

Management group likely affected growth of calves after birth due to different environmental conditions and age of calves at weaning. Differences in rainfall and feed availability between different years have been associated with differences in calf growth (Reynolds et al., 1990). Genetics of individual animals will also affect growth rates in different groups (Reynolds et al., 1990). Least squares mean weaning weights (Table 5) ranged from 219.2 to 272.0 kg among management groups.

### *Calf Losses*

*Abortions.* Abortion rates between 2 mo of pregnancy and term from 1,389 pregnancies were compared for treatment and management group. The arc sine transformation was used for ANOVA of percentages to determine statistical significance, but least squares means of untransformed data are presented. Abortion rates could not be determined for Trial 13 because 2-mo pregnancy data were unavailable. Abortion rates also were analyzed by fetal sex as determined by ultrasound for trials in which fetuses were sexed (1 through 10). An additional ANOVA was done on the subset of these in which overall fetal sexing accuracy (Table 6) was greater than 90% (Trials 1, 5, 7, 8, and 9).

There were no differences in abortion rates due to treatment ($P = 0.71$), or the interaction of groups and treatment ($P = 0.92$), although there was a management group effect ($P < 0.03$). Also, there was no difference in abortion rate due to fetal sex ($P > 0.10$) regardless of whether all data for fetal sex were used or just the subset of herds where fetal sex was evaluated with >90% accuracy. Least squares mean abortion rates presented in Table 4 are for all trials in which fetal sex was determined. Mean abortion rates were 4.0 and 2.4% for male and female fetuses ($P = 0.52$) in the subset of trials with 90% or greater ultrasound sexing accuracy. Conceptus losses between 1 and 2 mo gestation have been similar between sexed and control populations (Seidel et al., 1999).

Abortion rates can be influenced by environment, such as presence of locoweed, as shown by the variation among management groups. Least squares means ranged from 0.0 to 12.7% (Table 5). Bulls and other factors associated with AI may also influence abortion rates.

*Neonatal Deaths.* Neonatal deaths included calves born dead or that died within 24 h of birth. There was a difference in neonatal deaths ($P < 0.001$) among management groups, but no effect of calf sex, treatment, or interactions thereof ($P > 0.10$).

Major factors influencing survival at birth include illness, dystocia, and intensity of management. These factors likely explain the differences in neonatal death rates between management groups, which ranged from 0.0 to 17% (Table 5). Calves requiring assistance due to dystocia have higher rates of death than those not requiring assistance (Reynolds et al., 1990). It is possible that some deaths were due to overlooked abnormalities because calves were not studied intensively (e.g., by autopsying dead calves). It is unlikely that sexing sperm caused such abnormalities because death rates were not increased due to sexing sperm.

*Total Preweaning Deaths.* Total preweaning death rates include neonatal deaths plus deaths of calves that died more than 24 h after birth through the time of weaning. Differences were observed among management groups ($P < 0.001$), but no difference was observed for treatment or calf sex ($P > 0.10$; Tables 3 and 4).

Factors causing preweaning death include sickness and environmental differences, as seen in the different management groups. Environment greatly influences death rates between birth and weaning (Reynolds et al., 1990). Total preweaning death rates ranged from 0 to 18% for the management groups (Table 5).

### *Sex Ratios and Accuracy of Sexing*

Of the 1,169 calves born from sexed sperm, 954 resulted from insemination with X-sorted sperm, and 215 were from Y-sorted sperm. Sex was not recorded for 11 calves that were dead at birth. Fetal ultrasound sex could be assigned to five of these calves; for the remaining six, fetal sex was undetermined. There were 838 heifer calves born from X-sorted sperm, resulting in 87.8% accuracy. The Y-sorted sperm produced 198 bull calves with 92.1% accuracy.

**Table 6.** Sex ratios for calves born in each trial and fetal sexing accuracy

| Trial | Sexed % males[a] | No. of males | Control males, % | No. of males | Fetal sexing accuracy, % | | |
|---|---|---|---|---|---|---|---|
| | | | | | Males | Females | Overall |
| 1 | 5.3 | 1 | 37.9 | 11 | 100.0 | 97.3 | 97.9 |
| 2 | 17.2 | 5 | 64.0 | 16 | 80.0 | 72.1 | 73.6 |
| 3 | 16.0 | 4 | 56.4 | 31 | 70.8 | 70.4 | 70.5 |
| 4 | 14.9 | 7 | 47.4 | 9 | 75.0 | 92.0 | 87.9 |
| 5 | 90.0 | 36 | 33.3 | 5 | 89.1 | 100.0 | 90.9 |
| 6 | 16.6 | 27 | 45.3 | 29 | 79.2 | 84.9 | 84.1 |
| 7 | 48.3 | 58 | 59.3 | 48 | 94.9 | 94.3 | 94.6 |
| 8 | 8.7 | 9 | 48.5 | 32 | 97.6 | 99.2 | 98.8 |
| 9 | 4.0 | 1 | 50.0 | 12 | 81.3 | 100.0 | 93.9 |
| 10 | 75.0 | 27 | 52.4 | 22 | 94.1 | 64.9 | 78.9 |
| 11 | 48.8 | 61 | 46.1 | 35 | —[b] | —[b] | —[b] |
| 12 | 55.1 | 27 | 35.5 | 11 | —[b] | —[b] | —[b] |
| 13 | 11.9 | 46 | 48.5 | 129 | —[b] | —[b] | —[b] |
| Mean | 26.4 | 309 | 49.2 | 390 | 89.3 | 88.2 | 88.3 |

[a]Some farms wanted males, some wanted females, and some wanted males from some matings and females from other matings.
[b]Fetal sex not determined for Trials 11 to 13.

The 793 control calves resulted from unsorted sperm. Fetal ultrasound sex was used for three of six control calves dead at birth for which sex was not recorded. The sex ratio was 49.2% males; this is not different from the expected sex ratio for non-sexed offspring.

For Trial 13, there was no pregnancy diagnosis at 2 mo of gestation as in the other trials. Gestation length was used to determine which calves resulted from AI, and which resulted from rebreeding at a later estrus. Calves born within 290 d of insemination were assumed to be part of the study. The sex ratio of calves beyond this cut off (data not used) was 47.1% males, which is convincing evidence that the cut off used was appropriate and that nearly all calves included in the analysis were part of the experiment as their sex ratio was 11.9% males.

The purpose of sexing sperm is to produce more offspring of the desired sex. The majority of trials used X-sorted sperm to produce more heifers. Only Y-sorted sperm were used in Trial 5, whereas Trials 7, 10, 11, and 12 concerned both X- and Y-sorted sperm; the remaining trials used only X-sorted sperm. Table 6 shows sex ratios of calves born for each trial.

Ultrasound diagnosis of fetal sex was attempted in Trials 1 through 10, with a total of 945 calves designated as male or female. Technicians were not told whether sexed or control semen was used in each mating. Of the 320 fetuses determined to be male, 283 resulted in bull calves (89.3%). Of the 622 fetuses diagnosed as females, 545 (88.2%) were heifer calves at birth. Four calves from each of the fetal sex-diagnosed groups did not have sex recorded at birth. Two sets of male twins and one set of female twins were identified by ultrasound; all produced one calf of the diagnosed sex. Twin diagnosis was either incorrect or one of the fetuses was aborted. Those fetuses not diagnosed for fetal sex produced 161 bulls and 198 heifers, with five dead calves without recorded sex at birth. Overall fetal sexing accuracy by ultrasound was 88.3% (Table 6).

The lowest overall accuracies were observed for Trials 2 and 3. Technicians in these earlier trials were not as experienced as those in the later trials. Trials 4, 6, and 10 also show less than 90% overall accuracy. For Trial 4, there was low accuracy of sexing males, whereas in Trial 10, accuracy was low in sexing female fetuses. Fetal sexing accuracy approaching 100% is possible with properly trained personnel and appropriate facilities and equipment (Table 6).

## Implications

No difference between calves produced from sexed or control sperm was detected for any of the characteristics studied. In addition, no gross anatomical abnormalities occurred. A neurological disorder that was observed in five calves from one location likely was not caused by the sexing process because it was observed in both sexed and control calves. Some offspring produced during these trials were bred with sexed sperm to produce consecutive generations with sexed sperm, further supporting that there is little or no damage to offspring derived from sexed sperm. A more thorough study of offspring (e.g., autopsying dead calves) would allow for detection of abnormalities that might have been overlooked. Management group and calf sex caused differences in characteristics, as expected and reported in the literature. This study indicates that sexing sperm by flow cytometry can be approximately 90% accurate. Producers can be confident that sexed sperm will not result in increased abnormalities or affect calf characteristics.

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# Exploring the Role of Sexed Semen in Dairy Production Systems*

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## ABSTRACT

The availability of sexed semen in dairy cattle has been eagerly anticipated for many years, and recent developments in fluorescence activated cell sorting have brought this technology to the brink of commercial application. Two limitations still exist, namely sorting speed (hence, availability and price) and in vivo conception rates. Presently, about 150 to 200 straws of X-bearing sperm can be sorted per machine per day, and this comprises less that 0.5% of the daily domestic dairy semen requirement. Furthermore, conception rates in field trials involving virgin heifers have typically ranged from 35 to 40% with sexed semen, as compared with 55 to 60% for unsexed semen, and this limits the willingness of producers to pay a premium price for the product. The focus of this paper is to discuss 3 potential breeding schemes that can lead to widespread usage of sexed semen today, in spite of its aforementioned limitations. The first strategy, earlier (than normal) breeding of virgin heifers with sexed semen, followed by the use of unsexed semen for repeat services, would provide 62 to 66% female offspring at first calving with no detrimental effects on average age at calving or dystocia. Semen cost per heifer calf would increase by $47 to 56, $123 to 137, or $199 to 217, depending on conception rate, for sexed semen priced at $50, $100, or $150 per unit, respectively. The second strategy, in vitro embryo production using known donors with dairy heifers as recipients, could provide extra heifer calves at an added cost of $81 to 118 or $212 to 286 for female embryos costing $50 or $100, respectively. The third strategy, in vitro embryo production with anonymous donors and beef recipients, could increase net calf value per recipient by $52 to 97 (single transfers) or $171 to 240 (double transfers) for embryos costing $25 each and by $11 to 59 (single transfers) or $95 to 161 (double transfers) for embryos that cost $50. Thus, strategies exist for near-term application of sexed semen within the routine management of commercial dairy farms. Early adopters of this technology will capture economic benefits associated with extra replacement heifers, as well as enhanced biosecurity via the ability to expand rapidly from within a closed herd.
(**Key words:** sexed semen, replacement heifer, embryo transfer, in vitro production)

**Abbreviation key: ET** = embryo transfer, **IVP** = in vitro embryo production, **MAS** = marker-assisted selection, **MOET** = multiple ovulation and embryo transfer.

## INTRODUCTION TO SPERM SEXING TECHNOLOGY

Separation of bovine semen into fractions enriched for X-bearing sperm has been discussed for decades, and scientists have predicted a far-reaching impact of this technology. The technology would enhance producers' ability to obtain inexpensive replacement heifers; this would fuel "biosecure" herd expansions and mitigate some of the effects of high involuntary culling rates and poor reproductive efficiency. Altering the sex ratio could also increase the effectiveness of AI progeny testing programs, as well as the efficiency of multiple ovulation and embryo transfer (**MOET**) and in vitro embryo production (**IVP**) programs.

Various approaches for separating X- and Y-bearing sperm have been examined. Immunological sexing, i.e., killing the Y-bearing sperm, has been attempted in several studies (e.g., Bradley, 1989; Blecher et al., 1999), and one can imagine the creation of transgenic bulls that are able to produce only X-bearing sperm. However, the only proven method for separating male- and female-bearing sperm is the fluorescence activated cell sorting approach of Johnson et al. (1987a, 1987b, 1989, and 1999). Also known as the "Beltsville Sperm Sexing Technology," this method was subsequently licensed to XY, Inc. (www.xyinc.com) for commercial development. Because the bovine X chromosome contains 3.8% more DNA than its Y counterpart, sperm can be treated with DNA-specific fluorescent dye and subsequently sorted using high-throughput flow cytometry. However, many sperm are damaged, and a large proportion (e.g., 70%) remain unsorted. This procedure, although remarkably fast by "laboratory standards" is remarkably slow by "commercial standards." As noted by Johnson et al. (2000), speed of the sorting process has improved nearly

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50-fold in the past decade, and roughly 18 million sperm can now be sorted per hour. At this rate, up to 215 straws of X-bearing sperm (with 2 million sperm/straw) could be produced per machine in a 24-h period, but US dairy producers currently use approximately 43,825 units of dairy semen each day. Another 50-fold gain in speed is unlikely, given concurrent concerns about sperm damage and compromised conception rates.

The objective of this paper is not to forecast improvements in the efficiency of sperm sorting that will subsequently allow sexed semen to be used in conventional breeding programs. Rather, the objective is to develop and evaluate several "alternative" breeding strategies in which sexed semen technology can be implemented today, regardless of the aforementioned limitations in sorting speed and conception rate. In other words, this study seeks to explore commercial applications that can capitalize on the benefits of sexed semen, while respecting the current limits of the technology. The focus of this study is further limited to applications that will result in widespread use of sexed semen within the routine management system of commercial dairy farms, rather than niche applications that will be limited to a few selected pedigree breeders.

## REVIEW OF ITS IMPACT ON GENETIC PROGRESS AND HERD MANAGEMENT

Predictions in the scientific literature regarding the impact of sexed semen on genetic progress vary widely, as do projections of its impact on the profitability of dairy farmers. Van Vleck and Everett (1976) estimated that producers could afford to pay as much as $15.67 per unit for sexed semen, assuming an average cost of $6.00 for unsexed AI semen at that time. Later, Van Vleck (1981) revised this estimate up to $19.00 per unit of sexed semen and concluded that the rate of genetic progress in dairy cattle could increase by 15% if this product were widely available. However, later studies have predicted a much more modest improvement in genetic progress. Baker et al. (1990) suggested that the ability to alter the sex ratio of the offspring of elite sires and cows would have a very minor impact on the rate of genetic progress. However, they (Baker et al., 1990) predicted that sexed semen would have a major effect on the efficiency of farming systems, particularly in situations where male dairy calves have minimal (or perhaps negative) economic value. Montaldo et al. (1998) estimated that the rate of genetic gain in MOET nucleus schemes would increase by only 0.4 to 1.4% with sexed semen, although the availability of sexed semen would greatly enhance the efficiency of these programs by allowing the same rate of genetic progress with fewer transfers.

An additional advantage may be captured if the technologies of MOET, sexed semen, and marker-assisted selection (**MAS**) are combined. A limiting factor with respect to the application of MAS is the difficulty in producing more than 3 or 4 same-sex full-siblings using conventional embryo transfer (**ET**). For example, even if a particular elite sire or dam is heterozygous for a QTL of interest, one can easily end up with no male calves that possess the desired genotype, pass all necessary health tests, and produce viable semen. If the sire or dam is heterozygous for 2 or 3 different QTL, the chance of obtaining a healthy calf of the appropriate sex that has inherited all of the desired QTL alleles is negligible. Because the use of sexed semen would (nearly) double the size of each same-sex full-sib family, opportunities for MAS would be greatly enhanced.

The impact of sexed semen on herd management will likely be much greater than the corresponding impact on genetic progress. For example, using X-bearing sperm to mate virgin dairy heifers would substantially decrease the incidence of calving problems (dystocia), because female calves are smaller than males. In addition, the average lactation length of high-producing dairy cows could be extended to 18, 20, or even 24 mo, because these cows could easily provide their own replacements while averaging fewer than 2 calves per lifetime. Some authors, such as Van Vleck (1999) and Hohenboken (1999), have suggested that dairy producers could breed the top 50% of their cows, genetically speaking, using X-bearing dairy semen to produce replacement heifers. Meanwhile, the bottom 50% of their cows could be mated using Y-bearing beef semen to produce crossbred market steers. However, this strategy is unlikely to be adopted in practice for 2 reasons. First, the conception rates achieved with sexed semen in high-producing lactating cows will be unacceptably low, given the price of the product. Second, the increase in value of crossbred bull calves relative to their purebred counterparts will be virtually negligible relative to the potential value of the milk from their dams. Therefore, if a producer doesn't want heifer calves from a group of genetically inferior cows, a more sensible strategy would be to avoid breeding these cows altogether and instead extend their (terminal) lactations using bST.

Hohenboken (1999) discussed the potential benefits of using sexed semen in a crossbreeding scheme, albeit in beef cattle. In a terminal 3-breed cross, 20% of the population consists of purebred cows to produce purebred females, 24% consists of purebred cows to produce crossbred females, and 56% consists of crossbred cows to produce terminal market steers. With sexed semen, these proportions could change to 11, 13, and 76%, respectively, thereby increasing the number of market

**Table 1**. Summary of field trials conducted by XY, Inc., using sexed semen in Holstein heifers in Colorado.

| | | | Pregnancy rate | |
|---|---|---|---|---|
| Sperm type | Sperm unit | Placement | 30–33 d | 64–67 d |
| Sexed | 1.5 million | Uterine body | 57% (67/117) | 55% (64/117) |
| Sexed | 1.5 million | Uterine horn | 48% (27/56) | 41% (23/56) |
| Sexed | 3 million | Uterine body | 51% (58/114) | 46% (53/114) |
| Sexed | 3 million | Uterine horn | 55% (31/56) | 48% (27/56) |
| Unsexed | 20 million | Uterine body | 74% (87/118) | 69% (82/118) |

steers produced each year and, in turn, enhancing the profitability of the beef operation. Although crossbreeding is not popular among dairy producers today, added benefits may be attainable with sexed semen. For example, one could produce first cross ($F_1$) female Holstein × Jersey embryos (e.g., via IVP with anonymous donors) and implant these into Holstein heifers. In subsequent generations, additional $F_1$Holstein × Jersey embryos could be transferred into the existing $F_1$ Holstein × Jersey cows and heifers, thereby creating a continuous, terminal dairy crossbreeding scheme that would maximize heterosis while improving milk composition and reducing dystocia.

An additional, often overlooked, benefit of sexed semen is biosecurity. Commercial dairy herds are expanding rapidly in many states, as financial pressure leads producers to seek economies of scale. However, rapid expansion from within a closed herd is impossible without sexed semen or embryos. Therefore, most producers purchase cows and heifers from auctions, cattle dealers, or other farmers. When these animals arrive,the farm may be exposed to many new pathogens (e.g., leptospirosis, paratuberculosis). The rate of involuntary culling subsequently increases due to greater frequency of disease, inability of older cows to adapt to facilities, and poor genetic potential among purchased animals and their offspring. As a result, the producer needs more replacements, and the cycle continues. The availability of sexed semen would allow producers to expand from within a closed herd and avoid many of the aforementioned problems. Likewise, a cow-calf beef operation (on which the frequency of common "dairy" diseases may be low) could derive additional income by "renting" recipients to a neighboring dairy farmer who wishes to expand in a biosecure manner.

## RESULTS OF IN VIVO FIELD TRIALS

Numerous field trials with sexed bovine semen have been conducted within the past 5 yr in the United States, and most have used virgin heifers as mates due to concerns about conception rate. Results from several trials conducted by XY, Inc. in Colorado (Seidel et al., 1999) are summarized in Table 1. Mean pregnancy rates with unsexed control semen were 74% at examinations 30 to 33 d after insemination and 69% at reexaminations 64 to 67 d after insemination. Pregnancy rates with sexed semen at 30 to 33 d after insemination ranged from 48 to 55%, but the effects of sperm concentration (1.5 vs. 3 million per straw) and semen placement (uterine horn vs. uterine body) were minimal. Pregnancy loss between 30 and 33 d and 64 and 67 d after insemination with sexed semen ranged from 2 to 7%, similar to the rate observed with unsexed semen.

Results from 3 additional field trials in New York, California, and in both California and Texas are shown in Table 2 (Seidel and Shenk, 2002). Conception rates with unsexed control semen were 62, 61, and 43%, respectively, in these studies. In the New York study, sexed semen at concentrations of 1.5 million and 6 million sperm per unit gave conception rates of 43 and 41%, respectively. In the other 2 studies, low concentration (2 million sperm per unit) unsexed semen resulted in concentration rates of 49 and 46%, respectively, while low-concentration, centrifuged unsexed semen resulted in conception rates of 46 and 38%, respectively. Mean conception rate with sexed semen in both studies was 31%.

Results of an additional field trial in Wisconsin, in which conception rates with sexed semen were compared in herds with below average, average, or above average reproductive efficiency, are shown in Table 3 (Seidel and Shenk, 2002). Mean conception rate with unsexed semen was 58% across all herds, while conception rates with sexed semen were 21, 37, and 35% in below average, average, and above average herds, respectively.

Based on these results, it is clear that conception rates are compromised by semen sorting, at least at present, and strategies for in vivo commercial application should focus on approaches that provide cost-effective use of sexed semen without significantly extending average age at first calving.

## IN VITRO EMBRYO PRODUCTION WITH SEXED SEMEN

Because sorting speed (and, hence semen availability) is a major concern, one would hypothesize that

**Table 2**. Summary of field trials in Holstein and Jersey heifers by AI technicians and do-it-yourself (DIY) inseminators in commercial dairy herds.

| Location, date | Mates | Semen type | Sperm/unit | Conception rate |
|---|---|---|---|---|
| New York | Holsteins (N = 797) | Sexed | 1.5 million | 43% |
| Jul. '00–May'01 | | Sexed | 6 million | 41% |
| DIY inseminators | | Unsexed | 20 million | 62% |
| California | Jerseys (N = 637) | Sexed | 2 million | 31% |
| Jan. '01–Sep. '01 | Unsexed, centrifuged | | 2 million | 46% |
| AI technicians | | Unsexed | 2 million | 49% |
| | | Unsexed | 20 million | 61% |
| California, Texas | Holsteins (N = 513) | Sexed | 2 million | 31% |
| Apr. '01–Aug. '01 | Unsexed, centrifuged | | 2 million | 38% |
| DIY Inseminators | | Unsexed | 2 million | 46% |
| | | Unsexed | 20 million | 43% |

breeding programs based on IVP may be commercially applicable before corresponding in vivo applications. Lu et al. (1999) reported that a similar percentage of ova seem to be fertilized when using sexed or unsexed bovine sperm, but the rate of blastocyst production with sexed semen appears to be only about 70% of that obtained with unsexed semen. In addition, blastocyst production may be delayed slightly (about ½ day) in some cases. Nonetheless, their (Lu et al., 1999) field trial involving 106 double (i.e., twin) transfers of IVP embryos produced with Y-bearing sperm led to 41 live calves, of which 90% were male.

Wilson et al. (2003) reported the results of an IVP project with sexed semen on 7 commercial dairies in Wisconsin. Each month, farmers identified "genetically useful" cull cows from which they desired additional offspring (N = 88 donors). Ovaries were retrieved at slaughter, and the recovered ova were joined with sexed sperm from 3 young Holstein sires. Fresh embryos were transferred back into recipient cows and heifers on the same farms. An average of 3.8 transferable embryos were produced per donor cow, although only 2.7 were actually transferred due to large variation among donors and corresponding shortages in recipients. Mean pregnancy rates for IVP embryos were 18% in recipient cows (timed breeding) and 40% in recipient heifers (standing heats). Mean pregnancy rate from timed AI in control cows was 36%, so it appears that transferring IVP embryos into high-producing cows will not lead to acceptable pregnancy rates. Conversely, transferring IVP embryos into virgin heifers leads to the risk of severe calving difficulty in (rather rare) cases of extremely large IVP calves.

Amman (1999) observed that the ultimate use of the sexed semen is a critical consideration before sorting begins. For example, roughly 2 million sperm per unit are required to inseminate a dairy heifer in vivo, while <100,000 sperm are needed to fertilize 100 ova in an IVP program. Therefore, sperm could be sorted more slowly (perhaps with less damage) for use in IVP, and the semen could be packaged in straws containing 100,000 or fewer sperm per dose.

When producing sexed semen for use in an IVP program, one must decide whether to sort fresh semen (as is typically the case) or thawed, frozen semen. The latter approach has 2 limitations. First, relatively few X-bearing sperm can be isolated from a straw of thawed semen, after accounting for the killed, damaged, unsorted, and Y-bearing fractions. Second, thawed semen appears to have poorer staining qualities, and this may complicate efforts to separate X- and Y-bearing sperm. However, Lu et al. (1999) noted that sex ratios greater than 80:20 (in favor of either sex) could be obtained in an IVP program when thawed, frozen semen was subsequently stained and sorted using flow cytometry. Because so few sperm are needed for IVP applications, thawed semen may also offer some advantages. Semen availability would be greatly improved, in the sense that IVP

**Table 3**. Summary of a field trial conducted from February to June 2001 in Wisconsin involving 816 Holstein heifers. AI technicians, and herds with below average, average, or above average reproductive efficiency.

| Semen type | Sperm/unit | Herd reproduction level | Conception rate |
|---|---|---|---|
| Sexed | 2 million | Below average | 21% |
| Sexed | 2 million | Average | 37% |
| Sexed | 2 million | Above average | 35% |
| Unsexed | 20 million | All | 58% |

programs would gain access to sexed semen from a much wider variety of bulls. Furthermore, the need to sort an entire ejaculate of an elite dairy sire might be eliminated, thereby saving a significant "opportunity cost" (associated with killing or discarding a large proportion of this valuable semen).

While considering "custom sorting" applications, we should recognize that an 85:15 or 90:10 sex ratio may not be desirable in all applications. For example, in MOET nucleus schemes both male and female offspring of elite donor dams are needed. In this case, one may choose to alter sorting conditions such that a sex ratio of 70:30 or 65:35 is obtained. Furthermore, one may desire different sex ratios in different types of breedings within a MOET nucleus program. For example, the first flush of an elite yearling heifer might be used to produce male calves, such that the AI stud could begin rearing these calves to semen-producing age quickly, relative to the time at which their dams will receive their first genetic information. The second and third flushes could subsequently produce female calves that would be raised as potential replacement heifers in the next generation of the MOET herd. One concern of Amman (1999) was that the potential market for sexed semen to be used in IVP applications is quite small, such that this market might be satisfied by a single sorting machine. In that sense, creation of female IVP embryos might be considered a "niche" application. However, if it is possible to produce large quantities of IVP dairy embryos (e.g., using ovaries obtained from an abattoir), and if the demand for these embryos among beef and/or dairy producers is large, then sexing even a small amount of semen could have a potentially large impact on a dairy production system.

## ISSUES REGARDING COMMERCIAL IMPLEMENTATION

Are we finally on the verge of commercial application of sexed semen technology? By some accounts, we've been a bit premature in declaring this technology "commercially ready" in the past. For example, consider the "news" item reported in the October 1978 issue of Livestock Farming (page 50):

"*A company (name withheld) is offering a semen sexing service to cattle breeders, i.e., separation of semen into X and Y bearing fractions. It can be used on fresh or thawed semen. The cost of separation is $15–20 per ampoule, depending on the size of the order.*"

Had this semen sexing service materialized, numerous producers would have leapt at the chance to obtain sexed semen at such a modest price. However, this announcement was at least a quarter century ahead of its time, and current efforts to commercialize this technology should be cognizant of farmers' concerns about previous failures to deliver the product. Young et al. (1988) noted that the vast majority of dairy producers express keen interest in acquiring sexed semen, so one can hypothesize a high rate of adoption very quickly after this product becomes available. However, they (Young et al., 1988) also noted that the rate of adoption for many simple, inexpensive technologies that are widely known to enhance profitability, such as AI or milk recording, is far less than 100%.

What are the criteria for "successful implementation" of a new technology? The present paper relies on the following (perhaps restrictive) definition: a technology has been successfully implemented when it becomes integrated into the routine management of commercial dairy operations. By this definition, one can easily identify several "successes," including AI, recombinant bST, total mixed rations, timed breeding programs and evaporative cooling systems. By the same token, one can list several "failures," including ET and IVP (with aspiration of ova from live donors). The use of these technologies, up to this point, has been limited to a few highly marketable animals owned by a very small number of pedigree breeders.

Like many agricultural technologies, the ultimate beneficiary of sexed semen will be the consumer, who will continue to enjoy high-quality dairy products at affordable prices. Among agribusinesses and producers, early adopters of the technology will derive the greatest benefits, and widespread availability of X-bearing sperm and, hence, inexpensive replacement heifers, could eventually mitigate some of these benefits. Cost of the technology, including purchase of the sorting machine(s), maintenance costs, licensing fees, royalties, and additional labor, is a major concern. One company, Cogent (www.cogentuk.com), has begun offering sexed semen commercially. Cogent operates roughly a dozen sorting machines and sells X-bearing semen to dairy producers in Great Britain and several other countries. However, this company is currently not licensed to sell sexed semen in the United States. Results of this initial "commercialization experience" are difficult to quantify due to the lack of any scientific data or published results.

Given the limitations of sexed semen, primarily conception rate and sorting speed (hence, low availability and high price), we have 3 options. First, we can dwell on these limitations and wait for them to go away, if ever. In other words, we can let reproductive biologists "tweak" the current method (or develop a new method) that will provide conception rates comparable with unsexed semen and will produce thousands of units of sexed semen each day. Second, we can ignore these limitations and use sexed semen in the same way we

**Table 4**. Example of cost per heifer calf using sexed semen on every service (initial and repeat) for 1000 dairy heifers, according to conception rate and cost per unit of sexed semen. Calculations assume that unsexed semen costs $15 per unit and that sexed semen results in 85% heifer calves.

| Semen type | Conception rate | No. pregnant at service | | | Units of semen | Heifer calves | Cost per heifer calf | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 5 | | | $50 Semen | $100 Semen | $150 Semen |
| Unsexed | 60% | 600 | 936 | 990 | 1666 | 500 | $50 | $50 | $50 |
| Sexed | 45% | 450 | 834 | 950 | 2217 | 848 | $131 | $261 | $392 |
| Sexed | 40% | 400 | 784 | 922 | 2485 | 845 | $147 | $294 | $441 |
| Sexed | 35% | 350 | 725 | 884 | 2819 | 839 | $168 | $336 | $504 |

have used unsexed semen for decades. In other words, we can use this expensive semen carelessly, without making any substantial modifications to our breeding programs (we'll fail, of course, and then we'll conclude that the technology doesn't work). Third, we can recognize these limitations and develop new breeding strategies that are "tailor-made" for sexed semen. In other words, we can formulate programs that utilize sexed semen in an efficient, cost effective manner, even if the product is expensive and conception rates are compromised. Three such programs are described in the next section, but first let us review the results that we would expect by using sexed semen in a more "conventional" manner (i.e., by using sexed semen "across the board" on dairy heifers).

Table 4 illustrates the expected cost per heifer calf when sexed semen is used for all inseminations in virgin dairy heifers (i.e., first service and all repeat services), according to semen price and conception rate. Assuming 1000 heifers and a conception rate of 60% with unsexed semen, the number of pregnant heifers would be 600 after first service, 936 after third service, and 990 after fifth service. A total of 1666 units of semen would be used, and 500 heifer calves would be produced. Semen cost per heifer calf would be $50, assuming a per unit price of $15 for unsexed semen. Three different conception rates were considered for sexed semen: 45, 40, or 35%, and the assumed sex ratio was 85:15 in favor of females. Given these conception rates, the number of pregnant heifers after 3 services would be 834, 784, or 725, respectively, and the corresponding number of pregnancies after 5 services would be 950,922, or 884, respectively. The total number of units of sexed semen required would range from 2217 to 2819, depending on conception rate. At a conception rate of 45%, 848 heifer calves would be produced, and the additional cost per heifer calf (relative to controls) would be $81, $211, or $342 for sexed semen costing $50, $100, or $150 per unit, respectively. Likewise, a conception rate of 40% would lead to 845 heifer calves, and the additional cost per calf would be $97, $244, or $391, respectively. Lastly, a conception rate of 35% would lead to 839 heifer calves that cost an extra cost $118, $286, or $454 apiece, respectively. Therefore, the purchase of sexed semen could be justified at $50 per unit regardless of conception rate (35, 40, or 45%), but a cost of $100 per unit would be difficult to justify in this "conventional" scheme unless conception rates of 45% or greater could be achieved.

Extended age at first calving is also a major concern in breeding programs that utilize sexed semen for first and repeat services. As shown in Figure 1, only 38 (of 1000) heifers would calve later than 27 mo of age with unsexed semen, assuming a 70% heat detection rate and a minimum breeding age of 14 mo. When sexed semen is used for all inseminations; however, the number of heifers calving later than 27 mo of age increases to 103, 139, or 185 at conception rates of 45, 40, or 35%, respectively. The opportunity cost of extending the age at first calving for a significant proportion of heifers would negate much of the benefit of sexed semen in this conventional strategy, even if cost of the semen were modest.

## BREEDING STRATEGIES THAT CAN BE IMPLEMENTED TODAY

Amman (1999) suggested that the added benefit of sexed semen must be, at minimum, twice as large as

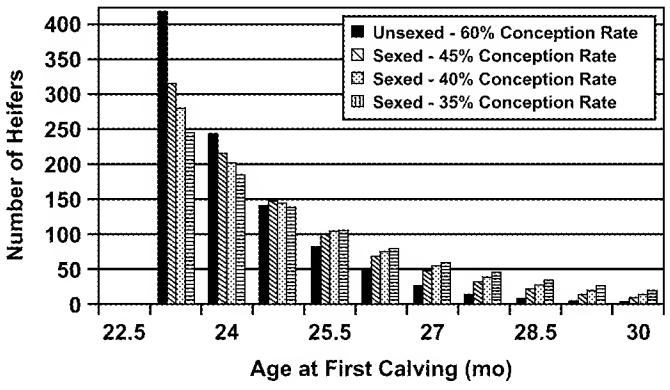


**Figure 1**. Expected distribution of age at first calving using sexed semen on every insemination (initial service and all repeat services) for 1000 dairy heifers, according to conception rate. Calculations assume a 70% heat detection rate.

**Table 5**. Example of cost per heifer calf using sexed semen on an early first service only (the "early bird" scheme) for 1000 dairy heifers, according to conception rate and cost per unit of sexed semen. Calculations assume that unsexed semen costs $15 per unit and that sexed semen results in 85% heifer calves.

| Semen type | Conception rate | No. Pregnant at service | | | Units of semen | Heifer calves | Cost per heifer calf | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 5 | | | $50 Semen | $100 Semen | $150 Semen |
| Unsexed | 60% | 600 | 936 | 990 | 1666 | 500 | $50 | $50 | $50 |
| Sexed | 45% | 450 | 912 | 986 | 1916 | 657 | $97 | $173 | $249 |
| Sexed | 40% | 400 | 904 | 985 | 2000 | 640 | $102 | $180 | $258 |
| Sexed | 35% | 350 | 896 | 983 | 1916 | 622 | $106 | $187 | $267 |

the added cost. In other words, if the cost of producing an extra heifer calf using sexed semen is $100 (after accounting for higher semen price, lower conception rate, and extended age at first calving), then an extra female calf should be worth at least $200 more than its male counterpart. Our discussion of in vivo applications of sexed semen, as well as in vitro applications that utilize dairy recipients, will focus on the expected cost per heifer calf, as compared with the cost of a heifer calf from unsexed AI semen. However, comparisons involving in vitro applications with beef recipients offer additional complications, because the relative value of female dairy calves is quite different from that of female beef calves, and because the option of double (twin) transfers exists. Therefore, these comparisons will be based on net calf value per recipient.

## The "Early Bird" Scheme

The "early bird" scheme is very simple, as shown in Figure 2. Virgin heifers are bred once (and only once) with sexed semen, and this breeding occurs 3 wk (one heat period) prior to implementation of the routine heifer breeding program. The underlying assumptions of this breeding scheme are as follows. First, conception rates with sexed semen are too low to justify the added semen cost (Table 4) and delay in age at first calving (Figure 1) that would occur if sexed semen were used at all services. Second, sexed semen will result in primarily heifer calves, and the corresponding reduction in calf size will offset (in terms of dystocia) any risks associated with calving 3 wk younger. And third, because sexed semen will be targeted for virgin heifers, AI companies will offer sexed semen from only those bulls that are known to transmit outstanding service sire calving ease (i.e., small calves).

Table 5 shows the expected cost per heifer calf in the "early bird" scheme. The minimum breeding age is reduced to 13.25 months, and sexed semen is used for first services only. All repeat inseminations rely on unsexed semen. A herd size of 1000 heifers is assumed, with a conception rate of 60% for unsexed semen, a heat detection rate of 70%, and a cost for unsexed semen of $15 per unit. As shown in Table 5, the number of pregnant heifers after 5 services is quite comparable with that of unsexed semen, and the number of units of semen required decreases dramatically, as compared with the plan described earlier, in which all insemina-

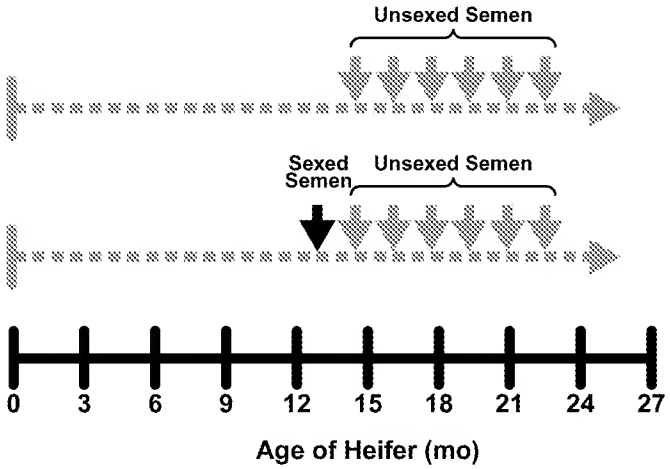


**Figure 2**. Schematic diagram of the "early bird" scheme.

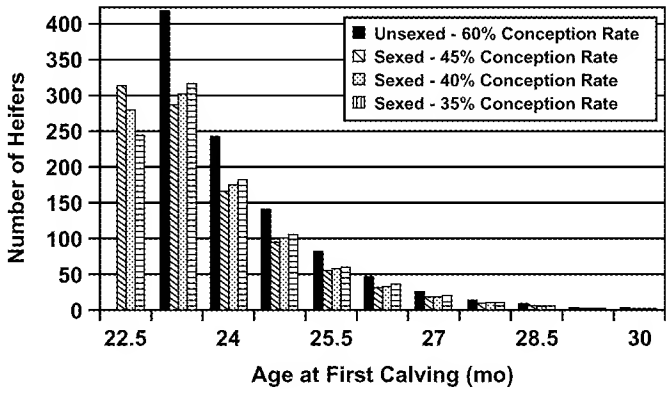


**Figure 3**. Expected distribution of age at first calving using sexed semen at an early first service only (i.e., the "early bird" scheme) for 1000 dairy heifers, according to conception rate. Calculations assume a 70% heat detection rate.

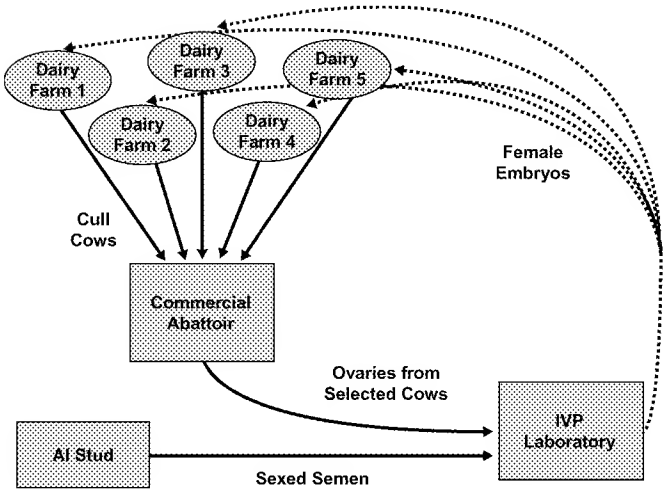


**Figure 4**. Schematic diagram of the "bovine recycling" scheme.

tions of heifers utilized sexed semen. The number of heifer calves produced by this scheme is 657, 640, or 622, corresponding to conception rates of 45, 40, or 35%, respectively. At a conception rate of 45%, the additional cost per heifer calf (relative to controls) is \$47, \$123, or \$199 for sexed semen costing \$50, \$100, or \$150 per unit, respectively. Similarly, the additional cost per heifer calf is \$52, \$130, or \$253, respectively, for a conception rate of 40%. Meanwhile, a conception rate of 35% would lead to an extra cost per heifer calf of \$56, \$137, or \$217, respectively. Thus, sexed semen could be justified at \$50 or \$100 per unit in the "early bird" scheme, but a cost of \$150 per unit could be justified only if the market price for heifer calves were extremely high. When sexed semen is used for an "early" first service, followed by resumption of the normal breeding program, the number of heifers with extended age at calving is slightly less than one achieves with unsexed semen only. As shown in Figure 3, unsexed semen leads to 38 (of 1000) heifers calving later than 27 mo of age, while sexed semen at an early first insemination, followed by unsexed semen for all repeat services, decreases this number to 29, 27, or 26 at conception rate of 45, 40, or 35%, respectively. Therefore, it appears that an "early bird" scheme, in which sexed semen is used for an early first service only, can provide positive economic returns by producing extra replacement heifers at an affordable price with no corresponding increases in age at first calving.

## The "Bovine Recycling" Scheme

The "bovine recycling" scheme combines IVP and sexed semen, as shown in Figure 4. Ovaries are retrieved at the time of slaughter from the best cows (genetically speaking) among all cows culled from a group of commercial dairies each month. If desired, the identity of the cow and her mate can be retained throughout the IVP and transfer process, such that the calves can be registered in a breed association herdbook. The underlying assumptions of this strategy are twofold. First, most dairy cows are culled for involuntary reasons (e.g., illness, injury, infertility, mastitis), and many of these "involuntary culls" are genetically suitable as dams of the next generation of replacement heifers. Second, the heritability values for most health and fertility traits are extremely low, so cows that are culled for these reasons are not necessarily genetically inferior for health or fertility (they may be subject to poor management or simply unlucky), and sire selection for health and fertility traits will offset any corresponding genetic inferiority among the donor dams.

An optimal IVP program that uses selected cull cows as donors will have a few key characteristics. Several large dairies must exist within reasonably close geographical proximity to each other, as well as to an abattoir and an IVP laboratory. Schedules for shipping animals to slaughter must be synchronized across herds, such that a large number of donors can be processed at one time. Managers of these dairies must agree to share the responsibility of providing donors and recipients, as well as the overall semen costs, laboratory fees, and transfer technicians. In this manner, variation in embryo production will "average out" across a large number of donors, and the total number of fresh, transferable embryos will closely match number of available recipients. Each farm may request "first right" to embryos from its own donors, but extra embryos must be transferred into any additional recipients that are available, because discarding IVP embryos is costly, as is leaving recipient animals open.

Table 6 shows the expected cost per heifer calf in an example "bovine recycling" scheme. Calculations assumed 10 cooperating 500-cow dairies with a 30% annual cull rate; 20% of the cull cows are assumed to be suitable dams of the next generation of replacement heifers. It was assumed that 3.8 transferable embryos could be produced per donor, in agreement with Wilson et al. (2003). Sexed semen was assumed to produce 85% female calves, and unsexed semen was assigned a price of \$15 per unit. Conception rates were 35% for AI inseminations, and 25, 20, or 15% for IVP transfers in milking cows. Corresponding conception rates in virgin heifers were 60, 45, 40, or 35%, respectively. The cost of producing female embryos (including laboratory fees, semen costs, and technician labor) was assumed to be either \$50 or \$100 per embryo.

As shown in Table 6, approximately 1140 transferable embryos could be produced annually, and transfers

**Table 6**. Example of cost per heifer calf using sexed semen and in vitro embryo production (IVP) with known donors (the "bovine recycling" scheme) on 10 cooperating farms of 500 cows each, according to conception rate and cost per unit of sexed semen. Calculations assume a 30% annual cull rate, 20% of cull cows selected as donors, 3.8 transferable embryos per donor cow, 85% heifer calves from sexed semen, and $15 per unit for unsexed semen.

| | | | | | | | | Cost per heifer calf | |
|---|---|---|---|---|---|---|---|---|---|
| Breeding type | Recipient type | Conception rate | No. culls | No. donors | No. embryos | No. pregnancies | No. heifer calves | $50 per embryo | $100 per embryo |
| Unsexed AI | Cows | 35% | | | | 399 | 200 | $86 | $86 |
| IVP Embryo | Cows | 25% | 1500 | 300 | 1140 | 285 | 228 | $250 | $500 |
| IVP Embryo | Cows | 20% | 1500 | 300 | 1140 | 228 | 194 | $294 | $588 |
| IVP Embryo | Cows | 15% | 1500 | 300 | 1140 | 171 | 145 | $393 | $786 |
| Unsexed AI | Heifers | 60% | | | | 684 | 342 | $50 | $50 |
| IVP Embryo | Heifers | 45% | 1500 | 300 | 1140 | 513 | 436 | $131 | $262 |
| IVP Embryo | Heifers | 40% | 1500 | 300 | 1140 | 456 | 388 | $147 | $294 |
| IVP Embryo | Heifers | 35% | 1500 | 300 | 1140 | 399 | 339 | $168 | $336 |

into virgin heifers would produce 94 or 46 extra heifer calves at conception rates of 45 or 40%, respectively. However, only 28 additional heifer calves would be produced in milking cows, even if the conception rate for IVP transfers were 25%. The added cost of producing a heifer calf by transferring female IVP embryos into milking cows, relative to the semen cost of $86 for an AI heifer calf, ranged from $164 to $700, so it is clear that IVP programs utilizing cows as recipients will be profitable only if conception rates are high (e.g., ≥ 25%) and embryo production costs are low (e.g., ≤ $50). On the other hand, the added cost per female calf using virgin heifers as recipients, as compared with the semen cost of $50 for an AI heifer calf, ranged from only $81 to $118 for embryos costing $50 each. However, the corresponding added cost ranged from $212 to $286 for embryos costing $100 each. Therefore, it appears that a "bovine recycling" program with known IVP donors and sexed semen can be profitable if embryos can be produced at a cost approaching $50 each and if conception rates of 40% or higher can be achieved. However, the risk of dystocia with IVP transfers into virgin dairy heifers must be considered, because the loss (death) of a few recipient heifers would negate most of the gains achieved by an altered sex ratio.

## The "Anonymous Donor" Scheme

As shown in Figure 5, the "anonymous donor" scheme once again exploits the synergies of sexed semen and IVP, but it also takes advantage of higher conception rates in beef recipients, as well as lower embryo production costs using ovaries from unknown donors that are obtained "in bulk" from a commercial abattoir. The key assumptions of this breeding program are as follows. First, an individual beef cow produces more than one (i.e., her own) replacement during her lifetime, such that extra gestation periods can be "borrowed" for transfers of dairy embryos. Second, female dairy calves have significantly greater value than female beef calves. Third, calving problems occur less frequently in beef cows and heifers, as compared with dairy (i.e., Holstein) heifers. Fourth, the seasonal nature of beef production leads to the availability of a large number of (synchronized) recipients for fresh IVP embryos on a given day. Fifth, conception rates in beef cows are quite high, so inserting one or two "rounds" of IVP transfers at the beginning of the breeding season will not substantially alter the seasonal calving pattern of a beef operation. And sixth, the genetic merit of dairy cows that are sent to commercial abattoirs in the Untied States is reasonably good, on average, such that intense selection among sires will offset any genetic mediocrity that might be encountered among dams.

An example of the expected net calf value per recipient in the "anonymous donor" program is shown in Table 7. It was assumed that a 500-cow beef herd would purchase IVP embryos and transfer these at first and

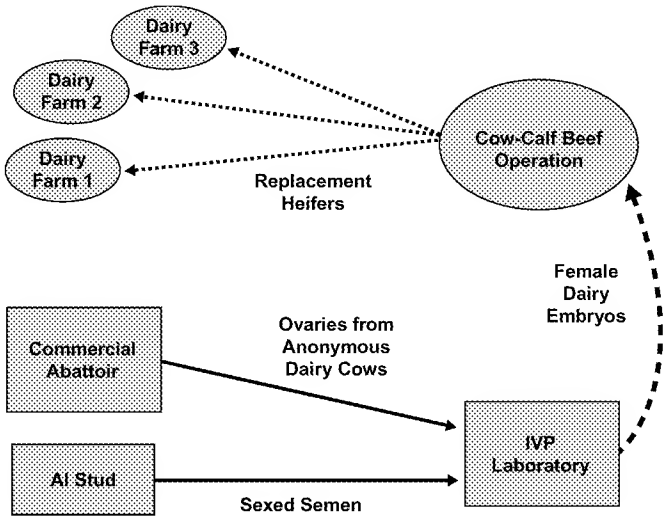


**Figure 5**. Schematic diagram of the "anonymous donor" scheme.

**Table 7**. Example of net calf value per recipient using sexed semen and in vitro embryo production (IVP) with anonymous donors (the "anonymous donors" scheme) in a beef herd of 500 cows. Scheme involves 2 transfers per recipient, followed by resumption of the normal breeding program. Both heifers and cows are used as recipients. Calculations assume $15 per unit for unsexed semen, 85% heifer calves from sexed semen, dairy heifer calf value of $400, dairy bull calf value of $100, beef heifer or bull calf value of $150, 33% increase in overall conception rate with twin transfers, and 50% twins among double transfer pregnancies.

| | | Round 1 | | Round 2 | | | | Net calf value/recipient | |
|---|---|---|---|---|---|---|---|---|---|
| Breeding type | Conception rate | No. transfers | No. pregnant | No. transfers | No. pregnant | Fertile heifers | Bulls and freemartins | $25 per embryo | $50 per embryo |
| Unsexed AI semen | 65% | 500 | 325 | 175 | 114 | 220 | 220 | $112 | $112 |
| Single IVP embryo | 45% | 500 | 225 | 275 | 124 | 297 | 52 | $209 | $171 |
| Single IVP embryo | 40% | 500 | 200 | 300 | 120 | 272 | 48 | $187 | $147 |
| Single IVP embryo | 35% | 500 | 175 | 325 | 114 | 426 | 43 | $164 | $123 |
| Double IVP embryo | 60% | 1000 | 300 | 400 | 120 | 482 | 180 | $352 | $283 |
| Double IVP embryo | 53% | 1000 | 267 | 466 | 124 | 449 | 168 | $319 | $245 |
| Double IVP embryo | 47% | 1000 | 233 | 534 | 125 | 411 | 153 | $283 | $207 |

(if necessary) second service for each cow and heifer. Calculations assumed a cost of $15 per unit for unsexed AI semen, 85% heifer calves from sexed semen, and calf values at birth of $400, $100, $150, and $150 for dairy heifer, dairy bull, beef heifer, and beef bull calves, respectively. It was further assumed that double transfers of IVP embryos would increase the overall conception rate by 33%, and that 50% of calvings resulting from double transfers would be twins. Conception rates were assumed to be 65% for unsexed AI semen, and 45, 40, or 35% for IVP embryos (before accounting for double transfers).

As shown in Table 7, insemination with unsexed AI semen (at the first 2 services) would produce 220 beef heifer calves, while the transfer of single IVP embryos would produce 297, 272, or 246 dairy heifer calves at conception rates of 45, 40, or 35%, respectively. On the other hand, transfer of double IVP embryos would produce 482, 449, or 411 fertile dairy heifer calves, respectively, after accounting for freemartins. Cost per fertile heifer calf (not shown) was slightly higher for double transfers than for single transfers, because the number of transfers increased twofold, while the conception rate increased only slightly. However, the key statistic in this production system is net calf value per recipient, and far more total heifer calves were produced via double transfers. As compared with a net calf value per recipient of $112 for conventional AI with unsexed beef semen, single IVP transfers produced an extra $52 to $97 per recipient for embryos costing $25 each and an extra $11 to $59 per recipient for embryos that cost $50 each. Double IVP transfers generated an extra $171 to $240 per recipient for embryos costing $25 each and an extra $95 to $171 per recipient for embryos that cost $50 each. Therefore, it appears that an "anonymous donor" scheme with sexed semen and unknown ova donors provides an excellent opportunity for entrepreneurial cow-calf operators to derive additional income by helping to satisfy the demand for dairy replacement heifers.

## CONCLUSIONS

Technology for sexing bovine sperm is indeed on the verge of commercial application. However, 2 key limitations exist: sorting speed and conception rate. The breeding strategies discussed herein, including the "early bird" scheme, the "bovine recycling" scheme, and the "anonymous donor" scheme, are ready for implementation today, in spite of the aforementioned technical limitations. Each program offers the potential for widespread application within the routine management system of "mainstream" commercial dairy farms, because each can provide additional female dairy calves at a cost that represents less that half of their added value. In this manner, herds can satisfy their need for high quality replacement heifers, and biosecure herd expansions become possible. "Niche" applications also exist, such as the use of sexed semen in MOET nucleus herds, but these should not be the primary goal. In all cases, the protocol for sorting semen should be tailored to the final application of the product, just as the breeding program should be adjusted to accommodate the benefits and limitations of sexed semen. Synergies with other tools and technologies, such as IVP, crossbreeding, MOET, and heat synchronization, will be the key to successful commercial implementation of sexed semen.

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| Application Number: | 09/879,480 |
| Filed: | June 12, 2001 |
| Title: | Integrated Herd Management System Utilizing Isolated Populations of X-Chromosome Bearing and Y-Chromosome Bearing Spermatozoa |

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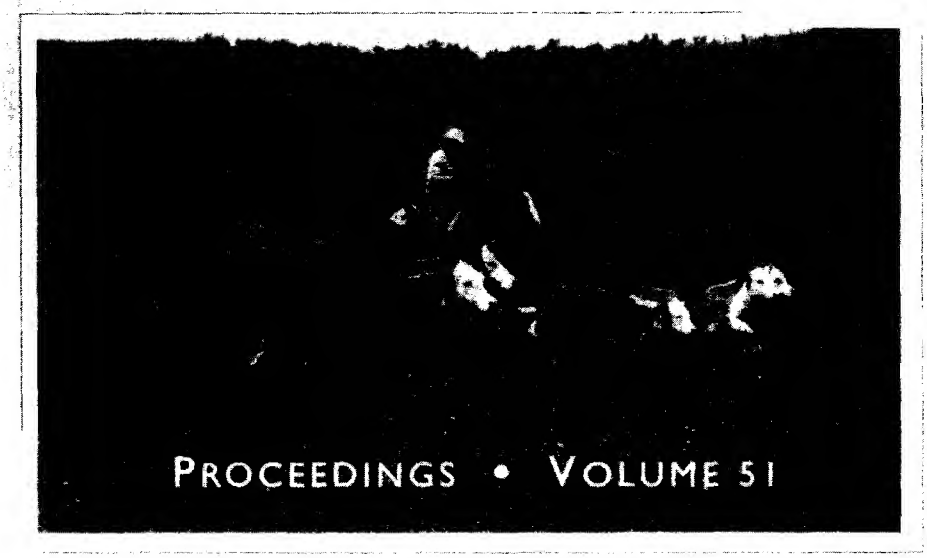
# Exhibit C

# Western Section
# American Society of Animal Science




## June 21–23, 2000

*University of California, Davis*

# PROCEEDINGS

# WESTERN SECTION
# AMERICAN SOCIETY OF ANIMAL SCIENCE

*VOLUME 51*

**Davis, California**
**June 21 – 23, 2000**

**RUMINANT NUTRITION**

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# Exhibit D

# ABSTRACTS
# AMERICAN SOCIETY OF ANIMAL SCIENCE
## WESTERN SECTION
**University of California-Davis**
**June 21–23, 2000**

*** Author Presenting Paper**

**1 Maternal biological efficiency in beef cattle.** J. D. Stevens*[1], D. D. Kress[1], D. C. Anderson[2], and K. C. Davis[1], [1]*Montana State University, Bozeman,* [2]*Northern Agricultural Research Center, Havre, MT.*

Three different breed groups of cattle were evaluated for maternal biological efficiency under range conditions. Breed groups were Hereford (HH), Tarentaise (TT) and the $F_1$ reciprocal crosses (HT). Seventeen females of each breed group were randomly chosen from a population of pregnant animals. Fecal output (FO) and forage intake (FI) were measured on 51 cows for each of 4 yr using a constant release intraruminal bolus of chromic oxide ($Cr_2O_3$). Following a 10-d waiting period to allow $Cr_2O_3$ to reach steady state equilibrium, three fecal grab samples were taken 3-d apart. Estimates were measured during five periods each year with PI in November, PII in February, PIII in May, PIV in June and PV in September. Calf weaning weight (CWW) was measured in October. Maternal biological efficiency was calculated for each cow/calf pair by dividing CWW by the sum of FI over the five periods (EFF) or by dividing CWW by the sum of FI over the five periods on a per unit body weight basis (EFF/BW). The same calculations were done for FO with very similiar results. Data were analyzed by the GLM procedures of SAS. The model contained dam breed group, age of dam (2, 3, 4 and 5+ yr), sex of calf, sire breed group and year. EFF and EFF/BW differed ($P<.05$) by cow breed group. Least-squares means for HH, HT and TT, respectively, were 6.64, 7.45 and 7.52 kg/kg for EFF and 3.46, 3.82 and 3.64 $kg \cdot g^{-1} \cdot kg^{-1}$ for EFF/BW. Linear contrasts for HH-TT showed the straightbreds were significantly different for EFF ($P<.05$) but not for EFF/BW ($P>.05$). The linear contrast for maternal heterosis indicated significant ($P<.05$) maternal heterosis for EFF/BW (7.6%) but not for EFF (5.2%). Age of dam was significant ($P<.05$) for EFF/BW but not for EFF. Least-squares means for 2-, 3-, 4- and 5 yr-olds were 3.02, 3.25, 3.52 and 3.82, respectively, for EFF/BW. Greater biological efficiency was exhibited by HT and TT cows than by HH cows under range conditions and maternal heterosis was positive for greater biological efficiency.

**Key Words:** Beef Cattle, Efficiency, Breed Differences

**2 Effects of season of use on beef cattle distribution patterns and subsequent vegetation use in mountain riparian areas.** C. Parsons*[1], P. Momont[1], T. DelCurto[2], and J. Sharp[2], [1]*University of Idaho, Caldwell,* [2]*Oregon State University, Union.*

To quantify the effects of season of use on beef cattle distribution relative to the riparian area, fifty-two cow/calf pairs were randomly assigned to two years of 3 replications of the following treatments: 1) early season (**ES**) grazing (mid-June to mid-July), and 2) late season (**LS**) grazing (mid-August to mid-September). Based on previous years DM production estimates, pastures were stocked to achieve 50 percent utilization after 28 days of grazing. Livestock observation points, livestock activities and ambient temperatures were recorded hourly during two 4-d periods in each season of use. Locations were then transcribed to a geographical information system (**GIS**) for the study area on Oregon State University's Hall Ranch in northeastern Oregon. Cow weight and body condition score (**BCS**), calf weight, ocular vegetation utilization estimates, forage quality, and fecal deposits within 1 m of the stream were recorded pre- and or post-grazing. During ES, cattle were further from the stream ($P < .01$) than LS cattle, averaging 161.4 and 99.4 m for ES and LS respectively. Grazing distribution also displayed a diurnal response ($P < .01$) with increasing ambient temperatures resulting in decreased cattle distance from the stream. Fecal deposits within 1 m of the stream tended ($P = .13$) to be greater following LS than ES grazing. Forage quality varied ($P < .01$) between seasons with ES forage having lower DM, greater CP, lower fiber, and greater IVDMD compared with LS forage. Livestock activity (grazing, ruminating or drinking) and grazing times, min/d, were not affected by season of use. However, forage utilization was influenced by season of use with ES grazing having lower riparian vegetation use and higher upland vegetation use as compared to LS grazing ($P < .05$). In summary, grazing season affected cattle distribution relative to the riparian area, with LS having more concentrated use of riparian vegetation.

**Key Words:** Season of Use, Beef Cattle, Distribution Patterns

**3 Profitability analysis of Holstein steers on various implant regimes.** S. L. Fowler*[1], J. Algeo[2], and J. L. Beckett[1], [1]*California Polytechnic State Unversity, San Luis Obispo,* [2]*Algeo Nutrition Consulting, Paso Robles.*

The purpose of this study was to determine the profitability of feeding Holstein steers using one of five implant regimes. One hundred seventy-six Holstein steers (144 kg) randomly assigned to one of five treatment groups (n = 36) were used to determine profit margin by implant treatment protocol. Treatments are listed in Table 1. Animals were weighed at 28-d intervals and weight gain, average daily gain (ADG), and feed efficiency were calculated. All implanted groups had heavier average final live weights, increased ADG and improved feed efficiency than non-implanted controls ($p< .05$). Steers were harvested after 291 d on feed, at which time carcass measurements were collected. Parameters used for determining profit margin include initial animal cost, feed, medicine,

the animals were slaughtered and the weights of their dissectable kidney (KAT) and subcutaneous adipose tissue (SCAT) were registered and samples of fat were used to quantify their proportion of fatty acids by HPLC using an online derivatization. Body weights were affected by the restriction (4.79 and 10.84 kg) . The proportion of C6, C8, C10, C12, C14, C16, C18, C18:1, C18:2, C18:3, C20, total saturated fatty acids and total unsaturated fatty acids in KAT were not affected by the restriction level. Only C16:1 was affected by the RL (0.41, 0.49 and 0.87% for RL100, RL80 and RL60, respectively, $P<0.08$). However in SCAT, C16:1 was in major proportion in the most restricted animals (0.3, 0.4 and 0.8 %, respectively, $P<0.05$) and the same was observed for C18:2 (1.39, 1.35 and 1.90% for RL100, RL80 and RL60, respectively, $P<0.09$). Saturated fatty acids were in lower proportion and unsaturated fatty acids were in higher proportion in RL60 animals ($P<0.08$). The RL affected body weight, the proportion dissectable adipose tissue and the profiles of fatty acids . The SCAT was the most affected.

**Key Words:** Fatty Acids, Undernutrition, Goats

**72 Influence of variation in supplement inclusion rate and laidlomycin propionate on growth performance of feedlot cattle.** R. A. Zinn[1], A. Plascencia*[2], M. F. Montano[2], and A. Pereira[1], [1]*University of California, Davis,* [2]*Universidad Autonoma, Baja California, Mexicali, Mexico.*

For many types of feed formulations a CV of 5% has become a standard for uniformity of feed mixing, permitting that an animal receives at least 90% of its formulated dietary allowances 95% of the time. However, with diets intended for feedlot cattle, the diversity of physical form and density of individual feed ingredients complicates the preparation of uniform feed mixes. Risks to cattle health and growth performance when uniformity in spacial distribution of critical ingredients is reduced has not been investigated. In this study, 112 yearling steers (364 kg) were used in a 123-d trial to evaluate the interaction of variation in supplement inclusion rate (0 vs 20% CV) and laidlomyin propionate (0 vs 10 mg/kg) on growth performance and carcass characteristics. The basal diet contained 71.8% steam-flaked corn, 3.0% cottonseed meal, 10.0% sudangrass hay, 3.5% yellow grease, 8.0% molasses, and 3.7% supplement. Weekly variation was produced by altering the inclusion rate of the supplement portion of the diet, as follows: on d 1, and d 5 of each week the diet contained 125% of the specified amount of supplement; of d 2, d 4, and d 6 the diet contained 100% of the specified amount of supplement; on d 3 and d 7 the diet contained 75% of the specified amount of supplement. Accordingly, the CV in weekly supplement concentration of the complete mixed diet was 20%. There were no treatment interactions ($P > .20$). Variation in supplement inclusion rate decreased ADG (8%;$P < .05$), gain efficiency (5%;$P < .01$), dietary NEm (2.3 %, $P < .01$), and dietary NEg (3.3%, $P < .01$). Supplemental laidlomycin tended to increase ADG (6%, $P = .19$), but did not affect ($P > .20$) gain efficiency of dietary NE. There were no treatment effects ($P > .20$) on carcass characteristics. We conclude that variation in supplement inclusion rate can have a marked effect on growth performance of feedlot cattle.

**Key Words:** Feed Mixing, Variation, Cattle

**73 Integration of early weaning and sexed semen into a single-calf heifer system to increase the value of non-replacement heifers.** B.A. Ereth*, J.C. Whittier, P.D. Burns, D.N. Schutz, D.W. Couch, and G.E. Seidel, Jr, *Colorado State University, Fort Collins.*

Integrating early weaning and sexed semen into a single-calf heifer system may be an alternative to traditional marketing of non-replacement heifers. This integrated system (IS) has potential for heifers to produce a calf by 20 mo of age and a carcass by 24 mo of age. Phase I of the IS included early weaning, estrous synchronization, and AI. Sexed semen was utilized to yield female progeny, decrease calving difficulty risk and create a second generation to perpetuate the IS. The IS group was composed of 46 Red Angus X Hereford heifers rejected for replacement status and early weaned at 110 ± 15.0 d. The control group was 40 traditional weaned (TW) heifers from the same herd, managed in a traditional replacement system (TRS). The early weaned (EW) heifers were placed on self-feeders and adjusted to a finishing ration. They were fed to 65% of mature weight to induce early puberty by 9 mo of age. At time of TW, age of EW heifers was less than TW heifers (202 ± 15.0 d and 229 ± 2.8 d respectively, $P < .0001$ ). There was no difference between groups in weight at TW, yet EW heifers had greater weight per day of age (WDA) than TW heifers, 1.2 ± .10 kg and 1.1 ± .11 kg ( $P < .0001$). Dams of EW heifers had greater body condition score (BCS), 6.6 ± .80 than TW dams, 5.8 ± .78 ( $P < .0001$ ) by TW. At AI of EW heifers, weight was greater for EW than TW heifers, 313 ± 28.0 kg and 293 ± 31.4 kg respectively ( $P < .0031$ ) and WDA of EW and TW heifers were 1.2 ± .10 kg/d and 1.0 ± .10 kg/d ( $P < .0001$ ) One month prior to MGA/PGF protocol synchronization, 20% EW and 8% TW were exhibiting estrous cycles as determined by progesterone assay. At fixed-time mating, age of EW heifers was 293 ± 15.0 d with 85% cyclic. Ultrasonography at 35-d post AI revealed first service conception rates of 27%. Phase I of the IS increased BCS of dams, enabled greater gains and weights of heifers, induced early puberty and resulted in 9 mo old heifers pregnant to sexed semen.

**Key Words:** Early Weaning, Single-Calf Heifer System, Sexed Semen

**74 Seasonal mineral status of beef cattle on twenty-two ranches across western Colorado evaluated using liver and serum concentrations.** B. Ereth, J. Whittier*, B. Toombs, R. Baird-LeValley, C. Mucklow, and M. King, *Colorado State University, Fort Collins.*

Twenty-two beef cattle herds across western Colorado were evaluated for trace mineral status. Serum and liver samples were taken twice yearly for two consecutive years to establish baseline levels of Cu, Fe, Mn, Mg, Mo, K, N. Estimated feed and water intake for each herd gave approximate total intake of each mineral. Cattle categorized as Cu deficient according to liver biopsy ( < 40 ppm ) was 70.84%, however, according to serum ( < .6 ppm ), only 25.58% were reported as deficient. Pearson® correlation coefficient between total serum copper and liver copper in year one was .339 ( $P < .0001$). These results indicate that serum was not an accurate measurement of copper reserves in beef cows. Both years exhibited similar trends and results comparing serum/liver viability. Mineral status changed from fall 93 to spring 94 (year 1) and from fall 94 to spring 95 (year 2) for all trace minerals reported except Mo. Liver concentration of Fe and Mn decreased from fall to spring in both years ( $P < .0002$ and $P < .0007$ respectively year 1, $P < .0001$ and $P < .0299$ year 2 respectively ). Liver concentration of Zn increased from fall to spring in both years 1 and 2 ( $P < .0001$ and $P < .0027$). Liver Cu concentration decreased from fall to spring in year 1 ( $P < .0155$ ) but increased in year 2 ( $P < .0001$ ). County of herd origin had an effect on liver concentration of all trace minerals for years 1 and 2. Mineral status of western Colorado varies between counties and from spring to fall with the most accurate measurement being liver concentration taken via liver biopsy.

**Key Words:** Beef Cows, Trace Minerals

**75 Effect of hay source and level on tissue trace mineral concentrations in growing steers.** E. E. Grings*[1] and W. W. Poland[2], [1]*USDA-ARS, Miles City, MT,* [2]*Dickinson Research Extension Center, North Dakota State University, Dickinson.*

Twenty-four steers (avg 410 kg) were fed alfalfa or western wheatgrass hay at either 1.90 or 2.35% of BW for 91 d to evaluate the effects of hay type and feeding level on tissue trace mineral concentrations. At the end of the feeding period, steers were slaughtered at a commercial abattoir. Tissue weights and samples were collected; samples were oven dried and analyzed for Cu, Zn, Mn, and Fe by atomic absorption spectrophotometry. Data were analyzed with a model including hay source and intake level, their interactions, and BW as a covariate. Alfalfa hay contained 7 ppm Cu, 17 ppm Zn, 30 ppm Mn, 426 ppm Fe, and 3.6 ppm Mo. Grass hay contained 2 ppm Cu, 20 ppm Zn, 29 ppm Mn, 143 ppm Fe, and 1.3 ppm Mo. Increasing hay intake by 124% increased ($P<.01$) intake of minerals but had little effect on tissue mineral concentrations. Heart Cu concentrations were greater ($P<.10$) in steers on the high than low intake level. Kidney Zn concentrations were affected ($P<.10$) by the hay source by level interaction. Liver weights averaged 1,466 g DM for alfalfa-fed and 1,276 g DM for grass-fed steers ($P<.01$). On a dry tissue basis, total liver Cu, Zn, and Mn were affected by hay source, being greater in alfalfa-fed than grass-fed steers (55.7 vs 31.0 mg Cu, 162.3 vs 139.5 mg Zn, 16.0 vs 13.2 mg Mn for alfalfa- and grass-fed steers, respectively). For Zn and Mn this was due to increased liver weight but for Cu it was related to both increased concentration ($P<.01$) and liver weight in alfalfa-fed steers. Heart Zn concentrations ($P<.10$) and total heart Zn ($P<.05$) were greater for grass-fed (80.3 ppm and 25.7 mg)

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